Regulation of the Amino Acid Transporter B^0AT1 Expression in Renal Epithelial Cells

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1. SUMMARY

Amino acids play a pivotal role in every living organism as they function as building blocks of proteins, signaling molecules, metabolic precursor, etc. Due to their multiple roles in the body, amino acids availability must be tightly controlled and relies on their transport across cellular membrane via amino acid transporters.

In the kidney proximal tubule and in the small intestine, the overall directional (re)absorptive transport of amino acids is mainly driven by accumulative transporters located in the apical membrane of epithelial cells. The best characterized luminal transporter for neutral amino acids is B^0AT1 (SLC6A19). Expression and function of B^0AT1 in the kidney have been shown to depend on the associated protein collectrin/TMEM27 (Danilczyk et al., 2006). To better understand the cellular and molecular mechanism underlying this interaction, we generated MDCK cells inducibly overexpressing B^0AT1 and/or TMEM27. Our data showed that TMEM27 increases B^0AT1 function by upregulating its surface expression. Interestingly, we found that the expression of the amino acid transporter and its accessory protein strongly decreased following subsequent cell culture passages. We tested the impact of cell culture medium amino acid concentrations on the transporter and found that high amino acid levels inhibit B^0AT1 protein abundance. In contrast, the expression of a control transgene remained stable. To test whether this loss was due to inappropriately high amino acid uptake, freshly transduced MDCK cell lines were cultivated either with physiological amounts of amino acids or with the high concentration found in standard cell culture media. Expression of exogenous transporters was unaffected by physiological amino acid concentration in the media. Interestingly, mycoplasma infection resulted in a significant increase in transgene expression and correlated with the rapid metabolism of L-arginine. However, L-arginine metabolites were shown to play no role in transgene expression. In contrast, activation of the GCN2 pathway revealed by an increase in eIF2α phosphorylation may trigger transgene derepression. In addition to the transgene regulation, we found that mycoplasma-induced arginine depletion dramatically affected MDCK epithelia, resulting in a decrease in cell number and

trans-epithelial electrical resistance corresponding in a delay in tight junction formation.

Taken together, high extracellular amino acid concentration provided by cell culture media appears to inhibit the constitutive expression of concentrative amino acid transporters whereas L-arginine depletion by mycoplasma induces the expression of transgenes via stimulation of the GCN2 pathway.

2. ZUSAMMENFASSUNG

Aminosäuren spielen in jedem lebenden Organismus eine Schlüsselrolle, da sie als Bausteine für Proteine, Signalmoleküle, metabolische Vorläufermoleküle, etc. dienen. Aufgrund ihrer vielfachen Funktionen im Körper muss ihre Verfügbarkeit stark kontrolliert sein und ist vom Transport durch Zellmembrane via Aminosäuretransportern abhängig.

In den proximalen Tubuli der Niere und im Dünndarm ist der gerichtete, (re)absorbierende Aminosäurentransport hauptsächlich durch akkumulative Transporter der apikalen Membran von Epithelzellen angetrieben. Der am besten charakterisierte luminale Transporter für neutrale Aminosäuren ist B^0AT1 (SLC6A19). Es wurde gezeigt, dass die Expression und Funktion von B^0AT1 in der Niere vom assoziierten Protein Collectrin/TMEM27 abhängig ist (Danilczyk et al., 2006). Um die zellulären und molekularen Mechanismen die dieser Interaktion zugrunde liegen genauer zu verstehen, generierten wir MDCK Zellen die induzierbar B^0AT1 und/oder TMEM27 überexprimieren. Unsere Daten zeigten dass TMEM27 die B^0AT1 Funktion durch Hochregulation seiner Oberflächenexpression steigert. Interessanterweise fanden wir heraus, dass sich die Funktion dieses Aminosäuretransporter und seines assoziierten Proteins sich mit steigender Zellkultur-Passage stark reduziert. Wir überprüften den Einfluss der Aminosäurekonzentrationen im Zellkulturmedium auf den Transporter und fanden heraus, dass ein hohes Aminosäure-Niveau zu einer Reduktion der Menge des Proteins B^0AT1 führt. Im Gegensatz dazu blieb die Expression eines Kontroll-Transgens stabil. Um zu überprüfen ob diese Minderung aufgrund einer unangemessen hohen Aufnahme von Aminosäuren zustande kam, wurden frisch transduzierte MDCK Zellen entweder mit physiologischen Mengen von Aminosäuren oder mit der hohen Konzentration, wie in Standard-Zellkulturmedium gefunden, kultiviert. Die Expression exogener Transporter blieb unbeeinflusst durch physiologische Aminosäurekonzentrationen im Medium. Interessanterweise hatte eine Infektion mit Mykoplasmen eine signifikant erhöhte Transgen-Expression zur Folge welche mit der schnellen Metabolisierung von L-Arginin korrelierte. Allerdings wurde gezeigt, dass Metabolite von L-Arginin keine Rolle für die Transgen-Expression spielen. Die Aktivierung des GCN2 Stoffwechselweges, welche durch

eine Zunahme der Phosphorilierung des eIF2α festgestellt wuden, könnte hingegen die Transgen-Derepression ausgelöst haben. Zusätzlich zur Transgenregulation fanden wir heraus, dass der Mykoplasmen-induzierter Arginin-Mangel das MDCK Epithel stark beeinflusst. Es führte zum Beispiel zu einer Verminderung der Zellzahl und des trans-epithelialen elektrischen Widerstands, wobei Letzteres einer Verzögerung der Bildung von Tight-Junctions entspricht.

Zusammenfassend konnten wir zeigen, dass eine durch das Zellkulturmedium bereitgestellte hohe extrazelluläre Aminosäurekonzentration die konstitutive Expression von Aminosäuretransportern zu inhibieren scheint, wohingegen durch Mykoplasmen verursachter L-Arginin-Mangel die Expression von Transgenen durch Stimulation des GCN2-Stoffwechselweges induziert.

3. INTRODUCTION

3.1 Anatomy and physiology of the kidney

The kidneys are essential organs for the body homeostasis. They function as filters, disposing the metabolic products and the toxins from the blood and excreting them through the urine. They regulate the body's fluid volume and maintain the electrolyte and acid-base balance. They secrete important hormones such as erythropoietin, renin and calcitriol.

The human kidneys are paired, bean-shaped and located in the retroperitoneal cavity. The functional unit of the kidney is the nephron ($8 \cdot 10^5$ - $1.2 \cdot 10^6$ in humans) which consists of the glomerulus and the tubule. The glomerulus is a tuft of capillaries from which the plasma filtrate is formed. The tubule consists of highly specialized epithelial cells which are responsible for converting the filtrate into urine by reabsorbing fluids, electrolytes and nutrients. The tubule is subdivided into several segments with different sequential function: proximal convoluted tubule (PCT), proximal straight tubule (PST), thin descending limb of Henle's loop (tDLH), thin ascending limb of Henle's loop (tALH), thick ascending limb of Henle's loop (TAL), distal convoluted tubule (DCT), connecting tubule (CNT), cortical collecting duct (CCD), outer and inner medullar collecting duct (OMCD and IMCD) (Fig.1).

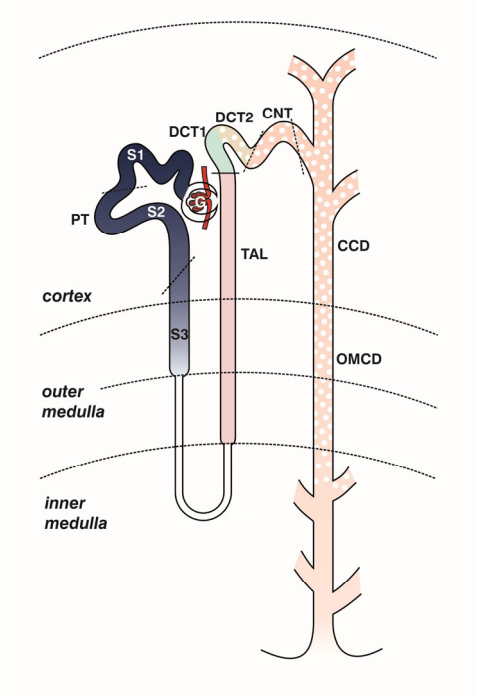

Figure 1. Organization of the nephron. The nephron comprises the glomerulus, which filters the blood, and the renal tubules, where reabsorption and secretion of ions and solutes occur. The tubule is subdivided in several segments. PT, proximal tubule; S1, S2 and S3, segments of the proximal tubule; TAL, thick ascending limbs; $DCT_{1/2}$, distal convoluted tubule; CNT, connecting tubule; CCD, cortical collecting duct; OMCD, medullary collecting duct. Figure taken from (Makrides et al., 2014)

The proximal tubules play a pivotal role in reabsorption of most of the nutrients (eg., amino acids and glucose), ions (eg., Ca^{2+}, HPO_4^{2-}), water and salts (NaCl and $NaHCO_3$), which all together represent the largest fraction of the glomerular filtrate.

3.2 Tubular amino acid reabsorption

Kidneys play an essential role in maintaining the body homeostasis, by tightly controlling the plasma levels of several nutrients which are present at low concentration in the body. Among those molecules, amino acids, which all together have a plasma concentration of ~ 2.5 mM, represent an interesting example (Verrey et al., 2005). Amino acids are derived from protein catabolism, *de novo* synthesis as well as intestinal absorption after a meal. Due to their small size, glomeruli are able to freely filter them and they would be lost in the urine if the tubular cells did not reabsorb them. Since amino acids are important for the human body, 98% of them are retrieved from the filtrate in the proximal tubules, corresponding to more than 50 g per day. Amino acid reabsorption occurs mostly in the first half of the proximal convoluted tubules (segment S1), where epithelial cells are equipped with a wide range of amino acid transporters on both cellular membranes (Fig. 2).

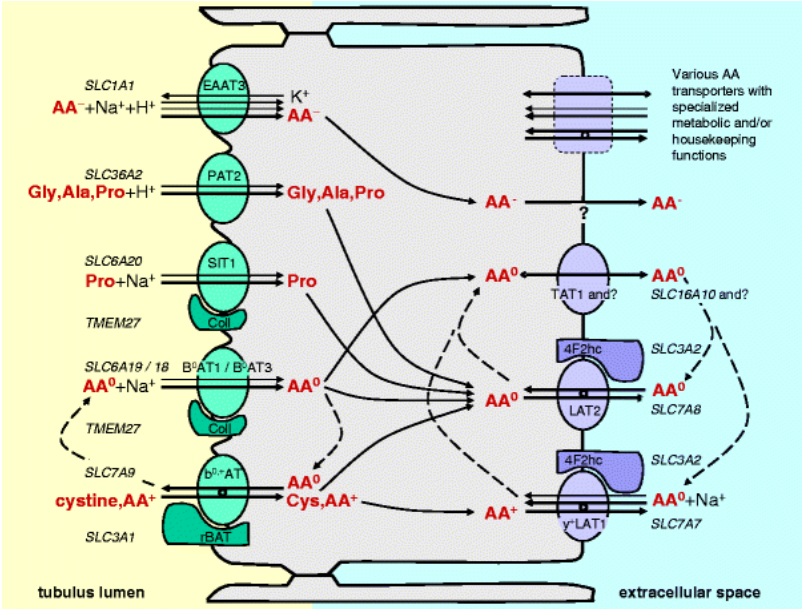

Figure 2. Schematic representation of the AA reabsorption in a renal proximal tubular cell. Amino acid transporters are indicated with SLC nomenclature and common names. Transported AAs are labeled in red. AA^0 neutral amino acid, AA^- anionic amino acid, AA^+ cationic, *Coll* collectrin/TMEM27. Figure taken from (Verrey et al., 2009).

At the apical membrane, amino acids enter the cells driven by the energetically favorable co-transport of Na^+ or H^+, as well as by exchangers. The exit from the cell is mediated by exchangers and facilitated diffusion pathways expressed on the basolateral membrane.

3.3 Amino acid transporters

Due to their complex nomenclature, transporters have been classified as Solute Carriers (SLC) in 52 families (http://slc.bioparadigms.org) based on their sequence homology. Among the members of the SLC families, there are about 50 genes coding for amino acid transporters grouped in 11 different families. In this thesis, I will focus on the neutral amino acid transporter SLC6A19 or B^0AT1 and its partner proteins TMEM27 and ACE2.

3.3.1 B^0AT1 (SLC6A19)

The neutral amino acid transporter B^0AT1 was first identified in 2004 in mice (Bröer et al., 2004), although evidences of its functional role had already been reported long before (Doyle and McGivan, 1992). B^0AT1 belongs to the family SLC6 of sodium- and chloride-dependent neurotransmitter transporters. It has been shown to localize in the brush border of the early proximal tubules (S1) and small intestine (Romeo et al., 2006). B^0AT1 function has been investigated through electrophysiological experiments in *Xenopus laevis* oocytes, which have shown an electrogenic transport of a broad range of neutral amino acids (Böhmer et al., 2005; Camargo et al., 2005). B^0AT1 has a low affinity for most of the neutral amino acids (K_m is in the low millimolar range) and its function does not depend on the presence of chloride, unlike for most other members of the SLC6 family.

Expression and function of B^0AT1 has been shown to depend on the co-expression of members of the Renin Angiotensin System (RAS), namely TMEM27 (collectrin) or angiotensin converting enzyme (ACE2) (Danilczyk et al., 2006; Camargo et al., 2009). The amino acid transporter interaction with its accessory proteins appeared to be organ specific. In the intestine, where TMEM27 is absent, B^0AT1 interacts with ACE2. In the kidneys although there is some expression of ACE2, B^0AT1 specifically interacts with TMEM27. In addition to ACE2 and

TMEM27, a novel interacting protein has been recently discovered in the intestine. The aminopeptidase N/CD13 has been shown to physically interact with the amino acid transporter *in vivo* and to increase B^0AT1 apparent affinity when co-expressed *in vitro* (Fairweather et al., 2012). B^0AT1 specific interaction with the associated proteins will be discuss in details in the next sections (3.3.2 and 3.3.3).

The human gene *SLC6A19* localizes to chromosome 5p15.33 and codes for a 634 amino acids protein. Mutations in the gene have been shown to cause an autosomal recessive condition, named Hartnup disorder, characterized by a neutral aminoaciduria (Kleta et al., 2004; Seow et al., 2004) (Fig. 3). In some cases, the urinary excretion of amino acids can be accompanied by pellagra-like symptoms including light-sensitive dermatitis, cerebellar ataxia, and other neurological or psychiatric symptoms (Henderson, 1958; Scriver, 1965; Bröer, 2009). Part of the phenotypic heterogeneity of Hartnup disorder may be due to the differential interactions of B^0AT1 mutants with the tissue specific accessory proteins (Camargo et al., 2009). Data obtained from *Slc6a19* null mouse confirmed an aminoaciduria consistent with the Hartnup disorder phenotype. The knockout eliminated both intestinal and renal expression of B^0AT1 although neither expression nor function of other neutral amino acid transporters was affected. Similarly, TMEM27 and ACE2 expression was not altered (Bröer et al., 2011).

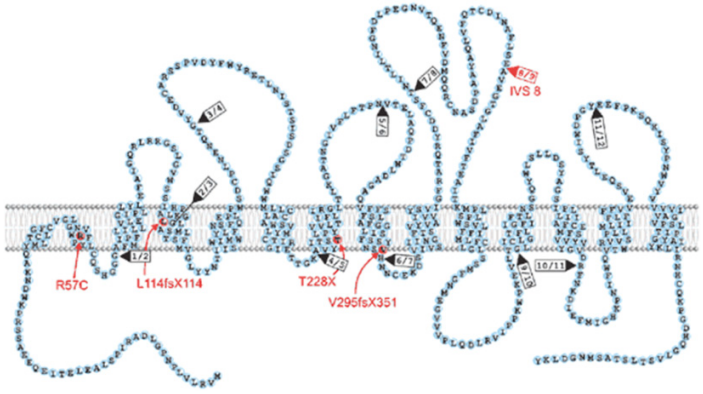

Figure 3. Depiction of B^0AT1 and Hartnup mutations. Mutations are indicated in red; arrowheads indicate splice sites. Figure taken from (Kleta et al., 2004).

The regulation of B^0AT1 has been extensively studied in different models such as *Xenopus laevis* oocytes and renal and intestinal cell lines *in vitro*, as well as various animal models *in vivo*. In addition to the already mentioned accessory

proteins TMEM27 and ACE2, which regulate the amino acid transporter expression, several kinases including the serum and glucocorticoid inducible kinases SGK1-3 (Böhmer et al., 2010), the Janus-activated kinase-2 JAK2 (Bhavsar et al., 2011), protein kinase B PKB/Akt and phosphatidylinositol-3 phosphate-5-kinase (PIKfyve) (Bogatikov et al., 2012) have been shown to upregulate B^0AT1 function when co-expressed in *Xenopus laevis* oocytes. Recent studies *in vitro* (Fanjul et al., 2012) and *ex vivo* (Ducroc et al., 2010) have suggested that leptin controls B^0AT1 activity via regulation of the protein traffic to the plasma membrane and modulation of the gene expression.

3.3.2 ACE2

ACE2 or ACEH was originally discovered as a homolog of ACE (Donoghue et al., 2000; Tipnis et al., 2000). The *ACE2* gene is mapped to the X chromosome and codes for an 805 amino acid type I glycoprotein. ACE2 is a zinc metallopeptidase oriented with the long N-terminus and catalytic site towards the extracellular space, where it can metabolize circulating peptides. Unlike somatic ACE, ACE2 contains only one active site and exhibits carboxymonopeptidase activity, cleaving a single C-terminal residue from peptide substrates. The carboxy-terminal domain of ACE2 is rather shorter and faces the cytoplasmic space. Interestingly, ACE2 shares about 42% identity with the amino-terminal domain of ACE (Donoghue et al., 2000) and about 48% identity with TMEM27 (Zhang et al., 2001). Therefore, ACE2 is considered a chimeric protein generated from the amino-terminal domain of ACE and carboxy-terminal domain of TMEM27 (Fig. 4).

ACE2 is highly expressed in the kidney on the apical surface of tubular epithelial cells, in the heart (both in the endothelium and in the cardiomyocytes), and in the adult Leydig cells of the testis ACE (Donoghue et al., 2000; Tipnis et al., 2000). Evidences of expression in the small intestine, lung, liver and placenta have been also reported (Hamming et al., 2007).

Three major ACE2 functions have been described so far (Fig. 5). The best known role of ACE2 is played within the RAS. While ACE cleaves angiotensin I (Ang I) to produce the potent vasoconstrictor angiotensin II (Ang II), ACE2 is a carboxypeptidase which converts angiotensin II (Ang II) to the vasodilator

heptapeptide angiotensin 1-7 (Ang 1-7). Therefore, ACE2 is often regarded as a negative regulator of ACE (Eriksson et al., 2002). In addition, ACE2 can also directly metabolize Ang I to generate angiotensin 1-9 (Ang 1-9) with lower efficiency than converting Ang II to Ang 1-7 (Corvol et al., 1995; Donoghue et al., 2000; Vickers et al., 2002).

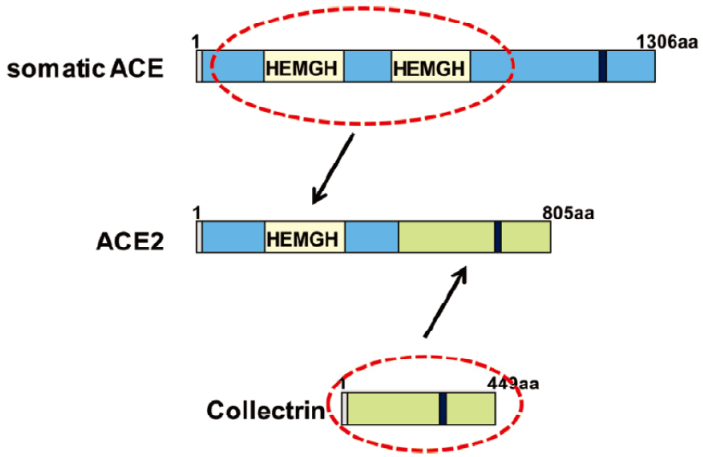

Figure 4. **The domain structure of somatic ACE, ACE2 and TMEM27 (collectrin).** Each protein is a type I integral membrane protein with a signal peptide (grey), and a transmembrane domain (black). The zinc-binding motif (HEMGH) repeats twice in ACE and once in ACE2, and is located within the homology region denoted by the yellow box. Regions of homology between ACE2 and TMEM27 are denoted in green, whereas homology of ACE and ACE2 is shown in blue. The numbers refer to the amino acids in each human protein. Figure taken from (Kuba et al., 2013).

The second role of ACE2 is played as severe acute respiratory syndrome corona-virus (SARS-CoV) receptor. Both *in vitro* (Li et al., 2003) and *in vivo* (Kuba et al., 2005) studies demonstrated that ACE2 mediates SARS-CoV infection through binding of the SARS-spike protein to its extracellular domain. The interaction of SARS-CoV with ACE2 leads to the endocytosis of virus particles through internalization with ACE2 and results in SARS-CoV infection. Although the receptor function does not require the peptidase activity of ACE2, the downregulation of ACE2 expression by SARS-CoV infection activates the renin angiotensin system and could explain the severe lung injury and acute lung failure typical in SARS pathology (Kuba et al., 2005). The third role of ACE2 is played as amino acid transporter partner protein. Studies by our group (Camargo et al., 2009) and others (Kowalczuk et al., 2008) demonstrated ACE2 co-localization with B^0AT1 at the

apical surface of the enterocytes in mice and human. In *Ace2* null mice, B^0AT1 expression is completely abolished in the small intestine, but normal in kidney. In addition, the physical interaction between the amino acid transporter and ACE2 has been confirmed by co-immunoprecipitation experiments *in vitro* and *in vivo* (Camargo et al., 2009). Similarly to its role as SARS-CoV receptor, ACE2 does not seem to require its carboxypeptidase activity to function as amino acid transporter partner protein. This theory is supported by *in vitro* studies which have shown that a catalytically dead ACE2 mutant was still able to promote B^0AT1 function (Camargo et al., 2009). Interestingly, *Slc6a19* null mice have not shown any decrease in ACE2 expression (Bröer et al., 2011). Furthermore, ACE2 is appropriately localized to membranes in organs in which the amino acid transporter is not expressed, consistent with the transporter having no impact on renin angiotensin system expression. In addition to B^0AT1, ACE2 has been shown to interact with SIT1 (SLC6A20) in the gut (Singer and Camargo, 2011), and B^0AT3 (SLC6A18) in the kidney (Singer et al., 2009). Recent studies (Hashimoto et al., 2012) have shown that *Ace2* deficient mice are more susceptible to experimentally induced colitis than the wild type littermates. This effect is due to an impaired amino acid absorption, which in turns affects the composition of the intestinal microbiota.

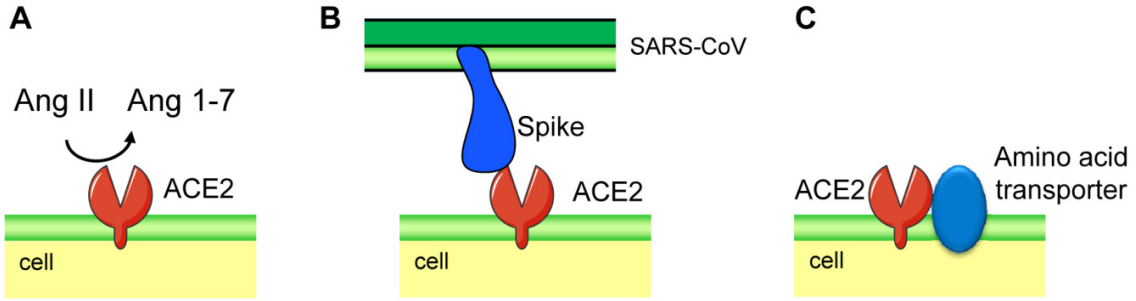

Figure 5. Multiple roles of ACE2. (A) ACE2 is a carboxypeptidase which converts angiotensin II (Ang II) to produce the heptapeptide angiotensin 1-7 (Ang 1-7). **(B)** ACE2 is a SARS-CoV receptor. **(C)** ACE2 interacts with amino acid transporters. Figure modified from (Kuba et al., 2013).

3.3.3 TMEM27

TMEM27 or collectrin was originally reported as a gene product (*NX-17*) upregulated in uninephrectomized mice (Zhang et al., 1999). Further analysis has revealed a gene localized on chromosome Xp22 which encodes a 222 amino acid protein (Zhang et al., 2001). Due to its close proximity with ACE2 on the genome and the 48% identity, TMEM27 and ACE2 have been proposed to constitute an ancient gene duplication (Chou et al., 2006). However, TMEM27 lacks the catalytic domain and it does not seem to play a crucial role in the RAS although an implication in the development of salt sensitive hypertension has been suggested (Yasuhara et al., 2008).

Expression of TMEM27 was initially restricted to the cytoplasm and apical membrane of renal collecting ducts, hence the name collectrin (Zhang et al., 2001). Further studies have demonstrated a broader expression in pancreatic β-cells and renal proximal tubules (Akpinar et al., 2005; Fukui et al., 2005; Danilczyk et al., 2006).

TMEM27 is a type I membrane protein with an N-terminal extracellular domain. It has two predicted N-glycosylation sites and runs at 43 kDa under reducing conditions. However, TMEM27 is also observed as a dimer of 90 kDa (Akpinar et al., 2005). It is a selective substrate of the beta site amyloid precursor protein cleaving enzyme 2 (Bace2). The activity of this sheddase leads to the cleavage of the 43-kDa TMEM27 into two products, a 25-kDa N-terminal fragment (NTF) which is released in the extracellular space, and a 22-kDa C-terminal fragment (CTF) remaining in the membrane. Interestingly, the CTF fragment is rapidly degraded by γ-secretase (Akpinar et al., 2005; Esterházy et al., 2011). Furthermore, the proteolytic activity of Bace2 has been shown to be selective for TMEM27 monomer but not for its dimer (Esterházy et al., 2012).

Analysis of *Tmem27* knockout mice by two independent groups has revealed the key role of Tmem27 as amino acid transporter partner protein (Danilczyk et al., 2006; Malakauskas et al., 2007). Both strains were characterized by a severe aminoaciduria denoted by a marked crystalluria which was due to the loss in the urine of nearly every amino acid, especially tyrosine and phenylalanine (Danilczyk et al., 2006; Malakauskas et al., 2007). *Tmem27* null mice were lacking the protein but

not the mRNA expression of several amino acid transporters in the proximal tubules. Further analysis has revealed a non-covalent interaction of TMEM27 with several amino acid transporters, including Slc6a19 (B^0AT1), Slc6a18 (B^0AT3), Slc6a20 (SIT1) and Slc1a1 (EAAT3). *In vitro* studies have shown that the function and surface expression of B^0AT1 is increased by the presence of TMEM27, suggesting a chaperone function (Danilczyk et al., 2006; Malakauskas et al., 2007). TMEM27 has been shown to bind to the N-ethylmaleimide-sensitive fusion attachment receptor (SNARE) complex proteins by directly interacting with snapin, a SNAP-25 binding protein (Fukui et al., 2005) Studies on EAAC1 have reported the crucial role of SNARE complexes in the transporter trafficking (Fournier and Robinson, 2006), supporting the role of TMEM27 as chaperone protein for B^0AT1.

The promoter of TMEM27 has been shown to be under control of the hepatocyte nuclear factor 1α and 1β (HNF-1α and 1β) (Akpinar et al., 2005; Fukui et al., 2005; Zhang et al., 2007). Interestingly, *HNF-1α* null mice were characterized by diabetes and a general defect in proximal tubules reabsorption consistent with the Fanconi syndrome (Pontoglio et al., 1996). Further localization of *HNF-1α* in renal proximal tubules and similar phenotype in *Tmem27* null mice have suggested that the expression of Tmem27 in proximal tubules is transcriptionally regulated by HNF-1α. In contrast to the proximal tubules, transcription of Tmem27 in the collecting ducts is controlled by HNF-1β. There, Tmem27 has been shown to play a role in primary cilium formation and cell polarity through vesicles transport mediation (Zhang et al., 2007). Tmem27 expression in pancreatic β-cells is also controlled by HNF-1α. However, its biological role in the endocrine pancreas is still debated. *In vivo* studies reported the importance of Tmem27 in insulin exocytosis (Fukui et al., 2005; Altirriba et al., 2010), pancreatic β-cell proliferation (Akpinar et al., 2005) and insulin sensitivity (Malakauskas et al., 2009). Surprisingly, *Tmem27* null mice exhibited no difference in pancreatic phenotype compared with wild-type animals but had altered glucose homeostasis (Malakauskas et al., 2009). However, the authors did not exclude that a compensatory mechanism by ACE2 or other proteins could mask the effect of Tmem27 deletion in the endocrine pancreas.

3.4 Amino acids

3.4.1 Structure

Amino acids are organic compounds characterized by an amino (-NH_2) and a carboxyl (-COOH) group bound to a central carbon (C). The side chain (R group) is what makes each amino acid unique. With the exception of glycine, all amino acids exhibit optical activity due to the presence of a chiral center (α-carbon atom) and they can have L- or D-isoforms (Fig. 6). More than 300 amino acids exist in nature; only 20 of them serve as building blocks for proteins and they are called proteinogenic amino acids. Accordingly to the international IUPAC nomenclature those amino acids are classified with a trivial name (i.e. alanine), a symbol code (Ala or A) and a systematic name (2-aminopropanoic acid) (1976). For convenience, in this work we refer to the 3-letter code or to the trivial name, always implying the L-isoform.

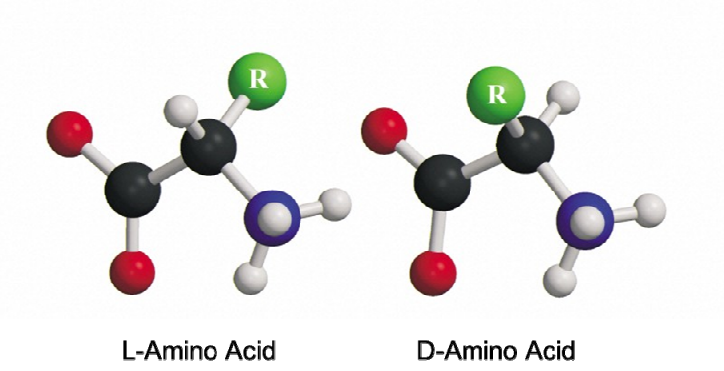

Figure 6. Amino acid chemical structure. Amino acids consist of an amino group (-NH_2), a carboxyl group (-COOH) and the side chain (R group) bound to a central carbon (α-carbon). H atoms in white, N atom in blue, C atoms in black and O atoms in red. Figure taken from http://wellcomeimages.org.

Due to their complexity and heterogeneity, amino acids have required several classifications. Nine amino acids (histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine) are defined essential amino acids as they cannot be synthesized *de novo* by the body and therefore have to be introduced through the diet. In contrast, non-essential amino acids are defined as

those amino acids which can be synthesized in adequate amounts by the body through the process of transamination. This classification is generally flexible as some amino acids can become conditionally essential in infants, during growth, premature birth or cirrhosis (Table 1).

Another classification of amino acids is based on the chemical structure of the side chain. Amino acids with an aromatic ring (phenylalanine, tyrosine and tryptophan) are defined as aromatic amino acids. Leucine, isoleucine and valine are classified as branched chain amino acids, due to their aliphatic side-chains with a branch.

Essential	Non-essential
Histidine	Alanine
Isoleucine	Arginine[a]
Leucine	Asparagine
Lysine	Aspartate
Methionine	Cysteine[a,b,c]
Phenylalanine	Glutamate
Threonine	Glutamine
Tryptophan	Glycine
Valine	Proline[a,b]
	Serine
	Tyrosine[c]

Table 1. Essential and non-essential amino acids. Conditionally essential amino acids during growth[a], premature birth[b] and cyrrosis[c]. Table modified from (Gropper, 2005).

3.4.2 Metabolism

In physiological conditions, plasma amino acid concentrations are tightly regulated and may vary within fixed limits (Cynober, 2002). After a meal, the liver takes up about 50-65% of the amino acids circulating in the blood and adjusts the rate of their metabolism according to the needs of the body (Gropper, 2009).

Amino acids are generally not used for energy production when carbohydrates or lipids are available to the organism. Instead amino acids are typically used to synthesize proteins and peptides, nitrogen atoms for the synthesis of other amino acids and nitrogenous or non nitrogenous compounds.

Although every amino acid undergoes a unique catabolic pathway, most of the amino acids follow a common route. The first step in amino acid conversion is the removal of the amino group from the amino acid through either transamination or deamination. The reaction of transamination involves the transfer of the amino group from an amino acid to an α-keto acid (the carbon skeleton) to form the analogous amino acid and to produce the α-keto acid of the original amino donor. This reaction is catalyzed by transaminases and it is an important step in the synthesis of non essential amino acids. The most usual and major α-keto acid involved in transamination is α-ketoglutarate. In contrast to the transamination, deamination reaction involves only the removal of an amino group, without direct transfer to another compound. The product of this reaction is another amino acid or an α-keto acid. Deamination reaction is carried out by specialized enzymes such as lyases, dehydratases or dehydrogenases. The toxic ammonia generated during deamination of amino acids will be safely removed from the system through the urea cycle. Once an α-keto acid is formed, it can be further metabolized and used to produce glucose, ketone bodies, cholesterol or fatty acids. The potential use of carbon skeletons depends on the amino acid it originates from but also on the physiological nutritional state of the body.

3.5 Amino acid sensing

Plasma amino acid concentrations can be challenged under physiological or pathophysiological conditions such as protein undernutrition, imbalanced diet or several forms of stress (trauma, thermal burn, sepsis, fever, etc.) (Chaveroux et al., 2010). In these conditions, higher eukaryotes have developed a finely tuned response in order to immediately adapt their metabolism accordingly. It is now well established that changes in amino acid availability, in addition to an endocrine and neuronal response, trigger cell-signaling events and regulate gene expression as well as transport and metabolism of amino acids themselves (Fafournoux et al., 2000; Kilberg et al., 2005; van Zeebroeck et al., 2008). Mammalian cells have two recognized pathways for controlling and responding to amino acid availability. Amino acid abundance in mammalian cells activates the mammalian target of rapamycin (mTOR) pathway that ultimately sustains anabolism and inhibits catabolism (Dennis et al., 1999; van Sluijters et al., 2000). In contrast, when amino acids are scarce, the general control nonrepressed 2 (GCN2) pathway is activated. As a result of this signaling cascade activation, general protein synthesis is decreased. Although these two pathways affect protein synthesis in opposite directions, it is not yet clear whether they are directly linked to each other (Kilberg et al., 2005).

3.5.1 GCN2 pathway

Eukaryotic cells sense amino acid deficiency through an accumulation of uncharged tRNAs which bind to and activate the GCN2 protein kinase (Berlanga et al., 1999; Sood et al., 2000; Zhang et al., 2002). GCN2 protein kinase phosphorylates and inactivates the eukaryotic initiation factor 2α (eIF2α), which in turn leads to a decrease of global mRNA translation (Kimball, 2002; Zhang et al., 2002). However, selected mRNAs with specific regulatory elements in their 5'-UTRs exhibit increased translation under the same circumstances. This phenomenon, known as translational derepression, was first described for the yeast transcription factor GCN4 (Hinnebusch, 1997), and then shown for the mammalian GCN4 homologue, activating transcription factor 4 (ATF4) (Harding et al., 2000). These transcription factors are able to bind to the Amino Acid Response Element (AARE)

sequences in the promoter region of selected genes and upregulate their transcription. Two sets of genes regulated by amino acid limitation have been identified: genes that are down- or up-regulated (Harding et al., 2003; Deval et al., 2009). Genes involved in regulation of transcription, signal transduction, lipid and carbohydrate metabolic processes, etc. are mostly downregulated (Deval et al., 2009). Among the genes which are upregulated by amino acid limitation, several ones encoding for amino acid transporters (*SLC7A1*, *SLC38A2* and *SLC7A5*), transcription factors (*ATF3*, *c-jun*, *C/EBPα*), and ribosomal proteins have been identified. The molecular mechanisms involved in the regulation of gene expression by amino acid limitation cover multiple steps along the pathway of DNA to RNA to protein (Kilberg et al., 2005). Beside the already mentioned transcriptional regulation through AARE sequences in the promoter region, two more main mechanisms of gene expression regulation by amino acid deficiency have been described. The first one involves stabilization of mRNA following amino acid limitation. Specific mRNAs present AU- rich elements (ARE) sequences in the 3'-untranslated region (3'-UTR) where regulatory proteins bind and control mRNA degradation or stabilization. Similar regulation has been described for the amino acid transporter CAT-1 (Yaman et al., 2002) and the activating transcription factor 3 (ATF3) (Pan et al., 2005). The third mechanism of gene expression regulation by amino acid deprivation involves the translational control of mRNA. The 5'-untranslated region (5'-UTR) of specific mRNAs contains upstream open reading frames (uORFs) which induce translation when cap-dependent protein synthesis is decreased. Examples of this mechanism have been observed for the activating transcription factor 4 (ATF4) (Vattem and Wek, 2004) and CAT-1 (Fernandez et al., 2002).

GCN2 activation is known to occur upon depletion of any essential, non essential or conditionally essential amino acids, whether this is due to dietary, enzymatic or pharmacological reasons (Gallinetti et al., 2013). GCN2 is only one of four kinases which target eIF2α and that collectively are activated by various cellular stresses (Fig. 7).

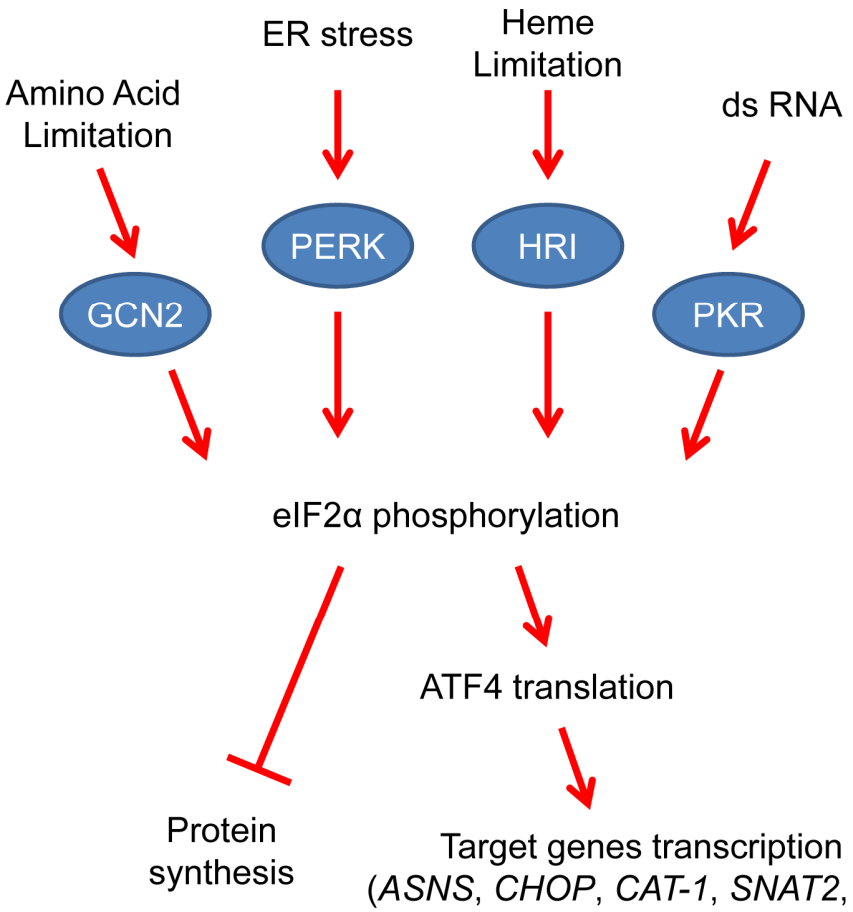

Figure 7. Amino acid response pathway. Several cellular stress signals activate the four eIF2α kinases GCN2, double-stranded RNA-activated protein kinase (PKR), double-stranded RNA activated protein kinase-like ER kinase (PERK), and heme-regulated inhibitor kinase (HRI), which in turn phosphorylate eIF2α and lead to a translational repression but a paradoxical increase in translation of ATF4. ATF4 upregulates the transcription of target genes encoding amino acid transporters (*SLC7A1* and *SLC38A2*), transcription factors (*CHOP*) or amino acid metabolic enzymes (*ASNS*). Figure modified from (Kilberg et al., 2005).

3.5.2 Amino acid signaling in mTOR pathway

The mammalian Target of Rapamycin (mTOR) is a highly conserved Ser/Thr kinase which plays a pivotal role in regulating cellular homeostasis and metabolism. Originally discovered in *Saccharomyces cerevisiae* as a direct result of the identification of its specific inhibitor rapamycin (Heitman et al., 1991), it is highly conserved from yeast to mammals. The mTOR kinase forms a part of two distinct complexes, mTORC1 and mTORC2. Although both complexes are stimulated by growth factors, only mTORC1 is sensitive to rapamycin and nutrients, including amino acids. Furthermore, they have separate downstream targets and functions.

Besides mTOR, mTOR complex 1 (mTORC1) comprises the regulatory-associated protein of mTOR (Raptor), the proline-rich Akt substrate 40 kDa (PRAS40), DEP domain-containing mTOR-interacting protein (Deptor), and the mammalian lethal with SEC13 protein 8 (mLST8). In contrast to the positively regulator mLST8 (Kim et al., 2003), both Deptor (Peterson et al., 2009) and PRAS40 (Sancak et al., 2007; Vander Haar et al., 2007) inhibit mTORC1 activity. Raptor is indispensable for substrate recognition but it is not yet clear whether it is a positive or negative regulator of mTORC1 activity (Yang and Guan, 2007).

In presence of favorable signals such as nutrients, growth factors and energy, mTOR promotes cellular growth by stimulating biosynthetic pathways, including protein synthesis. In contrast, during nutrient starvation, hypoxia or osmotic stress mTOR is inhibited and autophagy is promoted. Among the nutrients, amino acids play a pivotal role in mTOR activation, although it is currently unclear how amino acid sufficiency or limitation is sensed to modulate mTORC1 activity. Leucine, arginine and glutamine are the three most potent amino acids involved in mTOR activation (Hara et al., 1998; Nakajo et al., 2005). Amino acids are known to act directly through the Rheb small GTPase and independently on TSC1/2, a direct upstream regulator of Rheb (Roccio et al., 2006). Like other small GTPases, Rheb activation state depends on the GTP/GDP binding. The presence of amino acids favors a Rheb GTP-bound active state which is a potent activator of mTORC1 kinase activity. Alternatively, amino acid deprivation promotes Rheb-GTP hydrolysis, resulting in the inactive GDP-bound state. Recently, new molecular players in the upstream amino acid signaling have been identified at the lysosome surface and a

model has been proposed. Sabatini and coworkers have suggested that amino acids signal their availability from within the lysosome through the vacuolar H^+-ATPase (v-ATPase) (Fig. 8). Amino acid stimulation weakens the interaction between one of the v-ATPase subunit (V_1) and Ragulator, a novel protein complex which functions as guanosine exchange factor (GEF). Hence, Ragulator is able to activate another GTPase, named RAG GTPase. Once activated, the RAG complex recruits mTORC1 from the cytosol to the lysosome, where it becomes activated (Sancak et al., 2007; Sancak et al., 2008).

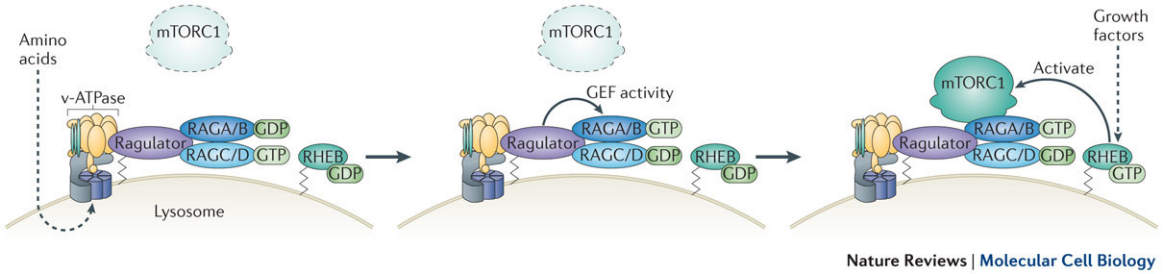

Figure 8. mTOR activation mechanism. Amino acids are sensed by the lysosomal v-ATPase, which controls the RAG GTPase-Ragulator binding and therefore Ragulator guanine exchange factor (GEF) activity. The active RAG complex, constituted of RAG A/B•GTP – RAG C/D•GDP binds to mTORC1 and recruits it to the lysosome, where RHEB can activate it. Figure taken from (Jewell et al., 2013).

The best studied mTORC1 downstream effectors are the translational regulators S6K1 (p70 ribosomal protein S6 kinase 1) and 4E-BP1 (eIF4E binding protein 1) (Fingar and Blenis, 2004). In their unphosphorylated state, both S6K1 and 4E-BP1 are bound to eIF3 (eukaryotic initiation factor 3) and remain inactive (Holz et al., 2005). Upon growth stimuli, mTORC1 binds to eIF3, phosphorylates S6K1 and 4E-BP1 and release them from the eIF3 complex. Once activated, 4E-BP1 and S6K1 enhance protein synthesis by increasing the initiation and progression of mRNA translation, respectively. Under basal conditions, 4E-BP1 binds and inhibits eIF-4E (eukaryotic translation initiation factor 4E). Upon phosphorylation by mTORC1, 4E-BP1 dissociates from eIF-4E, which in turn can bind the mRNA cap and initiate the translation (Haghighat et al., 1995; Hara et al., 1997). The active S6K1 also promotes mRNA translation by phosphorylating multiple key proteins involved in translation initiation and/or elongation (Jeno et al., 1988; Thomas, 2002).

3.5.3 Amino acid transceptors

Since amino acid transporters represent the natural bridge between cell and environment, there is growing recognition that they might have a role in nutrient sensing and signaling (Hundal and Taylor, 2009). Examples of amino acid transporters playing the double transporter/receptor role (so called “transceptors”) have been already described in lower eukaryotes such as *Saccharomyces cerevisiae* and *Drosophila melanogaster*. The yeast amino acid permeases Ssy1 (Didion et al., 1998; Klasson et al., 1999) and Gap1 (Donaton et al., 2003) are considered to function as sensor of extracellular amino acid availability since they both stimulate signaling pathways which evoke changes in metabolism and expression of a variety of stress-responsive genes. Two amino acid transporter genes have been identified in *Drosophila* as important regulator of growth and proliferation: *slimfast* (Colombani et al., 2003) and *path* (Goberdhan et al., 2005). The major example of transporter-like sensor in mammalian cells is represented by SNAT2 (SLC38A2), a member of the APC transporter superfamily to which Gap1, Ssy1, *slimfast* and *PATH* belong (Jack et al., 2000). Two independent lines of evidence indicate a potential role for SNAT2 in amino acid signaling. The increase in SNAT2 gene expression and surface localization in response to amino acid deprivation suggest an adaptive regulation of the amino acid transporter which may help to adjust the intracellular amino acid pool accordingly to extracellular amino acid availability (Gazzola et al., 2001; Hyde et al., 2001). Secondly, SNAT2 has been shown to affect insulin signaling through phosphoinositide 3-kinase (PI3K), therefore affecting the downstream mTOR pathway (Evans et al., 2008). This study suggests that SNAT2 may be able to signal directly to mTOR and modulate the intracellular amino acid concentrations.

Two mechanisms by which amino acid transporters may generate an amino acid-dependent cell signaling have been proposed (Hyde et al., 2003). In the first mechanism the amino acid transporter itself functions as a sensor and directly responds to changes in substrate availability generating cellular signaling. Alternatively, the amino acid transporter may affect the local amino acid concentrations and therefore modulate the initiation of signaling by intra- or extra-cellular receptors situated in its proximity.

3.6 Arginine

Among the 20 proteinogenic amino acids, arginine represents an interesting molecule. In healthy adults, arginine can be synthesized *de novo* from glutamine and citrulline. However, in certain conditions, as for example renal or intestinal dysfunction and inflammation, or in newborns and growing children, arginine must be introduced in the diet. Thus, arginine is classified as a conditionally essential amino acid (Flynn et al., 2002).

3.6.1 Arginine metabolism

The appearance of arginine in plasma in the fed status is both exogenous (diet) and endogenous (protein catabolism and endogenous synthesis). Plasma levels of arginine in healthy adults are ~ 80-120 µmol/L and are maintained fairly constant by the organism (Morris, 2007). The anabolic and catabolic pathways in which arginine is involved will be discussed in more details in the next sections.

3.6.1.1 Arginine anabolism

De novo arginine synthesis in adult animals and humans is negligible compared to the endogenous arginine flux deriving from protein catabolism (Wu and Morris, 1998). Arginine synthesis is the result of an interorgan communication between intestine and kidney known as intestinal-renal axis (Wu and Morris, 1998). In healthy adults, glutamine, glutamate and proline are converted into citrulline in the enterocytes (Wu and Morris, 1998). Plasma citrulline bypasses the liver without being taken up and reaches the renal proximal tubules where it is reabsorbed and converted into arginine by argininosuccinate synthetase (ASS) and argininosuccinate lyase (ASL) (Wu et al., 2007). The kidney is the major organ involved in arginine production as the renal arginine synthesis accounts for approximately 60% of the net arginine synthesis. In newborn and young infants, however, arginine synthesis occurs already at the level of the intestine. Due to an increased capacity of the kidney to synthesize arginine from glutamine, the intestinal synthesis of arginine is gradually decreased during development (Hurwitz and Kretchmer, 1986; Morris et al., 1991; Wu and Knabe, 1995).

The highest rate of arginine synthesis occurs in the liver where arginine is synthesized via the urea cycle. However, the net arginine synthesis by the liver is negligible because hepatocytes express high arginase levels which hydrolyze arginine into urea and ornithine (Wu and Morris, 1998).

3.6.1.2 Arginine catabolism

In contrast with the single anabolic pathway, arginine breakdown appears to be very complex, due to the large systemic and subcellular compartmentalization (Wu and Morris, 1998). Four enzymes are using arginine as substrate: arginase, nitric oxide synthase (NOS), arginine decarboxylase and arginine:glycine amidinotrasferase. The action of these enzymes results in production of several low molecular weight molecules which can be further metabolized (Fig. 9).

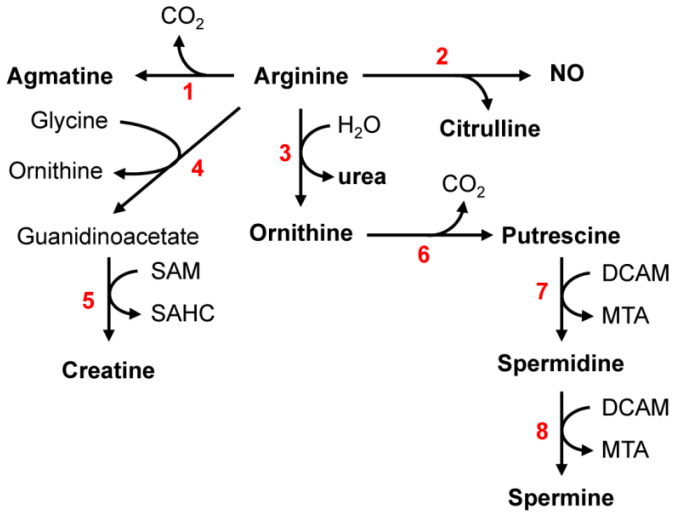

Figure 9. Pathways of arginine catabolism. Enzymatic pathways are schematic and not all substrate intermediates are shown. Enzymes which catalyze the reactions are: 1. arginine decarboxylase; 2. NOS; 3. arginase; 4. arginine:glycine amidinotransferase; 5. guanidinoacetate *N*-methyltransferase; 6. ornithine decarboxylase; 7. spermidine synthase; 8. spermine synthase. Abbreviations: SAM, *S*-adenosylmethionine; SAHC, *S*-adenosylhomocysteine; DCAM, decarboxylated *S*-adenosylmethionine; MTA, methylthioadenosine. Figure modified from (Wu and Morris, 1998).

Arginase. In mammals, the hydrolysis of arginine to ornithine and urea by arginase is quantitatively the most important pathway for arginine catabolism (Wu et al., 2009). Two isoforms of arginase exist and they are encoded by separate genes. Type I arginase is mostly expressed in the liver where it plays a major role in the urea cycle. Type II arginase is completely absent from the liver but has low expression levels in kidney, brain, small intestine, mammary gland and macrophages (Morris et al., 1997). In contrast to the cytosolic localization of type I, type II arginase is a mitochondrial enzyme and its subcellular compartmentalization might be a mechanism for regulating the metabolic fate of arginine, as already shown in enterocytes (Davis and Wu, 1998).

NOS. The family of nitric oxide synthases has been for the past several years the best known group of arginine-metabolizing enzymes, mostly thanks to the diverse roles played by nitric oxide (NO) as signaling molecule (Wu and Morris, 1998). NOSs convert arginine into NO and citrulline in a relatively low percentage compared with overall arginine catabolism (Wu and Morris, 1998). There are three distinct isoenzymes of NOS, encoded by separate genes: type I or neuronal NOS (nNOS), type II or inducible NOS (iNOS) and type III or endothelial NOS (eNOS). The constitutive nNOS and eNOS are primarily expressed in neurons and endothelial cells, respectively. Expression of iNOS can be induced by bacterial endotoxin and inflammatory cytokines in a wide range of cells and tissues (Pautz et al., 2010). In contrast to the Ca^{2+}-independent iNOS, activity of the constitutive NOS isoenzymes is highly regulated by Ca^{2+}/calmodulin.

Arginine decarboxylase. The conversion of arginine to agmatine is mediated by arginine decarboxylase. Originally described in plants and bacteria, arginine decarboxylase is expressed in the mitochondria of several mammalian cell tissues, including brain, liver, kidney, adrenal gland, macrophages and gut (Li et al., 1994; Morrissey et al., 1995; Sastre et al., 1998). Agmatine has been shown to play a role as cell signaling molecule (Li et al., 1994), anti-proliferative agent (Satriano et al., 1998) and endogenous regulator of NO synthesis (Galea et al., 1996).

Arginine:glycine amidinotrasferase. The first reaction in creatine synthesis is mediated by arginine:glycine amidinotransferase, which transfers the guanidine group from arginine to glycine to originate guanidinoacetate and ornithine. The

expression of this enzyme is mostly predominant in kidney and pancreas, although it has been shown to be localized in the liver and other organs as well (McGuire et al., 1986). Guanidinoacetate is methylated by guanidinoacetate *N*-methyltransferase to form creatine, which is transported in the blood to be used by skeletal muscles and nerves as energy source.

Ornithine decarboxylase. Polyamines are small molecules playing an essential role in cell proliferation and differentiation (Pegg, 1986). The most important polyamines are putrescine, spermidine and spermine. The synthesis of putrescine occurs through decarboxylation of ornithine by ornithine decarboxylase (ODC). ODC is considered the rate-limiting enzyme in the polyamine metabolism and it is regulated by hormones and growth factors (Janne and Raina, 1969; Bello-Fernandez et al., 1993). Spermidine is synthesized by spermidine synthase through the addition of the amino propyl group of the decarboxylated S-adenosylmethionine (DCAM) to putrescine. Subsequently, spermine is synthesized after fusing spermidine with another amino propyl group, DCAM, to spermidine.

3.6.2 Renal arginine transport

Reabsorption of arginine in the kidney involves specific amino acid transporters on the apical and basolateral membranes of proximal tubular cells. The apical uptake of arginine from the tubular fluid is mediated by the heterodimeric amino acid transporter $b^{0,+}$AT-rBAT. The light subunit $b^{0,+}$AT (SLC7A9) is covalently linked by a disulfide bond to the heavy subunit rBAT (SLC3A1) (Pfeiffer et al., 1999a; Wagner et al., 2001). $b^{0,+}$-rBAT functions as a high affinity antiporter, which exchanges large neutral amino acids (especially cystine) against dibasic (arginine, lysine, ornithine) amino acids (Chillarón et al., 1996). Defects in the transporter are known to cause cystinuria, an autosomal disease characterized by elevated urinary cystine, arginine, lysine and ornithine. Inheritance can be dominant or recessive and mutations can affect both *$b^{0,+}$AT* and *rBAT* genes (Calonge et al., 1994; Chillarón et al., 2010).

Arginine transport across the basolateral membrane is mediated by the system y^+, mainly by CAT1 (SLC7A1) and y^+LAT1-4F2hc (SLC7A6-SLC3A2). CAT1 was originally described as the receptor for murine ecotropic leukaemia retrovirus (Kim et al., 1991). It mediates Na^+-independent transport of cationic amino acids and it is

considered to be the major system y^+ transporter in most cells (Verrey et al., 2004). CAT-1 expression is regulated by amino acid limitation (Hyatt et al., 1997). Removal of a single essential amino acid or all amino acids induces an increase of CAT-1 protein expression through transcriptional and posttranscriptional mechanisms (Hatzoglou et al., 2004). CAT-1 gene shows an AARE sequence within the first exon of the genomic sequence which mediates the transcriptional activation upon amino acid deprivation (Fernandez et al., 2003). Furthermore, regulatory proteins are known to associate with CAT-1 mRNA and stabilize it (Yaman et al., 2002). In addition to the transcription upregulation and the increase of mRNA stability, CAT-1 translation is also regulated following amino acid deprivation. The internal ribosome entry sequence (IRES) in CAT-1 mRNA allows an m^7G-cap independent translation initiation, resulting in an increase of CAT-1 protein synthesis (Fernandez et al., 2001; Fernandez et al., 2002).

y^+LAT1 and 4F2hc form a heterodimeric amino acid transporter which mediates the efflux of cationic amino acids in exchange for neutral amino acids and Na^+ (Pfeiffer et al., 1999b; Fukasawa et al., 2000). Mutations in y^+LAT1 cause lysinuric protein intolerance, an autosomal recessive disease characterized by low plasma and elevated urine concentrations of cationic amino acids. Because y^+LAT1 is expressed in both the intestine and the kidney, defects in its function result in an impaired intestinal absorption and renal reabsorption. Patients manifest also hyperammonemia, caused by an impaired urea cycle, and develop an aversion to protein-rich foods (hence the name) (Borsani et al., 1999; Torrents et al., 1999).

The tertiary active function of $b^{0,+}$AT-rBAT and y^+LAT1-4F2hc relies on the parallel function of the apical B^0AT1/SLC6A19 and basolateral TAT1/SLC16A10, respectively. In fact, the obligatory antiporters $b^{0,+}$AT-rBAT and y^+LAT1-4F2hc recycle the neutral amino acids transported by B^0AT1 and TAT1 in exchange for cationic amino acids.

Since the kidneys are the main site of arginine *de novo* synthesis from citrulline, the transport of this amino acid is crucial for arginine homeostasis. The renal tubular citrulline uptake is most likely mediated by the apical B^0AT1 and $b^{0,+}$AT-rBAT (Mitsuoka et al., 2009) and by the basolateral OAT1 (SLC22A6) (Nakakariya et al., 2009).

Figure 10 summarizes the major arginine, citrulline and ornithine transporters and the metabolic pathways in which arginine is involved in renal epithelial cells.

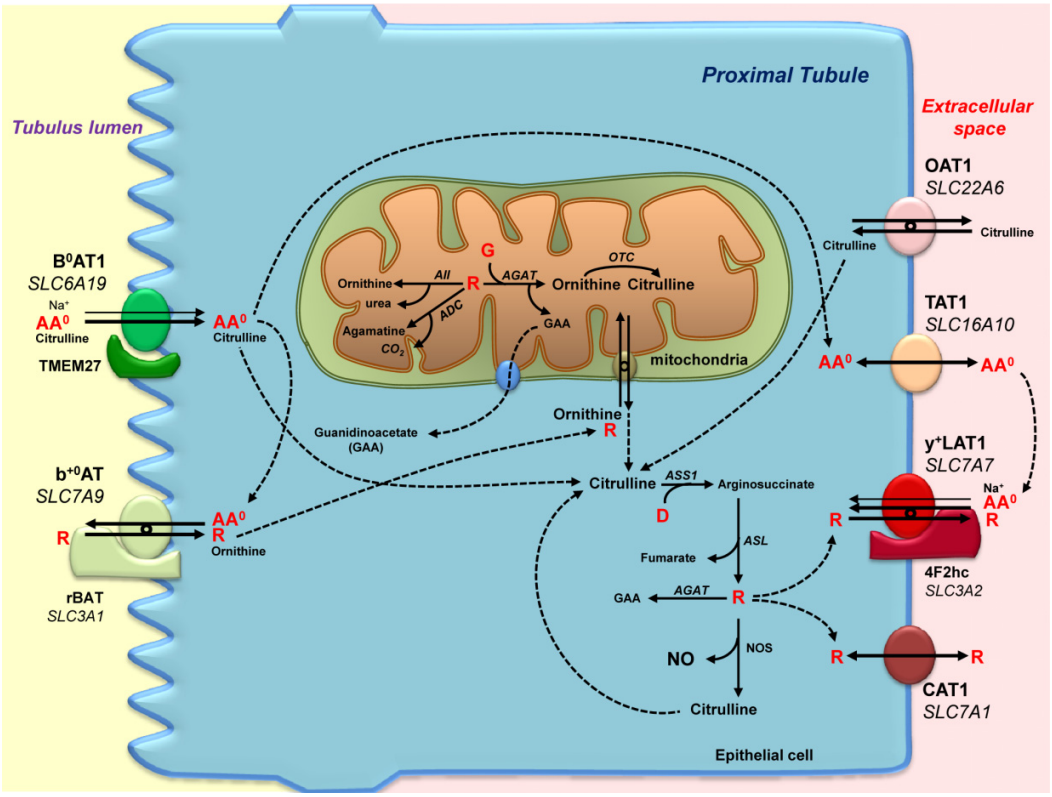

Figure 10. Schematic overview of the crosstalk between arginine, citrulline and ornithine transporter and their metabolism in renal epithelial cells. Apical b^{0+}-rBAT transporter uptakes L-arginine (R) in exchange for neutral amino acids (AA^0), driven by the transport of B^0AT1, which takes up neutral amino acids as well as citrulline. On the basolateral membrane OAT1 likely mediates citrulline uptake. R efflux is mediated by y^+LAT1-4F2hc in exchange for aromatic AA^0, which are likely recycled by the facilitated diffusion pathway TAT1. Additionally, R may be exported by basolateral CAT1. In the cytoplasm, citrulline is converted to R via the sequential action of arginosuccinate synthetase (ASS) and arginosuccinate lyase (ASL). R is converted back to citrulline by NOS, which in addition generates nitric oxide (NO). In the mitochondria, R is metabolized by different pathways. Metabolism of R by arginine:glycine amidotransferase (AGAT) produces ornithine and guanidinoacetate (GAA). This reaction can also occur in the cytoplasm and the GAA is released in the blood stream for creatine production. R can be decarboxylated by arginine decarboxylase (ADC) to agmatine and CO_2. Mitochondrial argininase II hydrolyzes R to produce ornithine. Ornithine can be converted to citrulline by ornithine transcarbamoylase (OTC). Enzymatic pathways are schematic and not all substrate intermediates are shown. Dotted lines indicate either translocation of substrates, eg., through the cytoplasm or extracellular space, or multiple metabolic steps. Figure taken from (Makrides et al., 2014)

3.7 Thesis projects

1. Role of TMEM27 for the function and surface expression of B^0AT1 in renal epithelial cells

Here we characterize the cellular and molecular mechanisms underlying the interaction between the amino acid transporter B^0AT1 and the accessory protein TMEM27 in the kidney. An MDCK cell line overexpressing B^0AT1 and TMEM27 was chosen as *in vitro* model for our study.

2. Role of amino acids for the regulation of B^0AT1 expression in renal epithelial cells

Here we investigate the impact of amino acid concentrations on the expression of the amino acid transporter B^0AT1 and the accessory protein TMEM27 in MDCK cells. The results of this study are summarized in the manuscript entitled “Amino acids regulate transgene expression in MDCK cells”.

4. B^0AT1-TMEM27 INTERACTION PROJECT

4.1 Materials and methods

4.1.1 Cell lines

The Madin Darby Canine Kidney (MDCK, strain I) cell line was used as *in vitro* model for our studies. MDCK cells derived from the distal tubules of canine kidney and they grow as tight polarized monolayers when cultured on porous filter substrates (Simmons, 1981). Cells were grown in Dulbecco's modified Eagle's medium (DMEM; catalog no. E15-810, GE Healthcare) supplemented with 10% heat-inactivated FBS (Sigma-Aldrich #F7524), 2 mM L-glutamine and 1% non essential amino acids (NEAA; catalog no. M11-003, GE Healthcare) at standard cell culture conditions (37°C, 95% relative humidity and 5% CO_2). The growth medium was changed every second day and the cells were splitted (1:10 to 1:20) two times per week. To allow cell polarization, cells were seeded at confluent density ($1.7 \cdot 10^5$ cells/cm^2) and cultivated on Corning Costar Transwell filters for 6 days in standard cell culture medium.

Human embryonic kidney cells (HEK293), kindly provided by Dr. D. Schümperli (University of Bern) were grown in DMEM (catalog no. E15-810, GE Healthcare) supplemented with 10% heat-inactivated FBS (Sigma-Aldrich), 2 mM L-Glutamine and 1% non essential amino acids at standard cell culture conditions (37°C, 95% relative humidity and 5% CO_2). Upon reaching 80-90% confluency, cells were trypsinized and passaged at a density of 10%.

4.1.2 cDNA constructs, transfection and lentiviral transduction

All vectors required to produce lentiviruses were designed by the Tronolab (EPFL, Lausanne). The envelope plasmid pMD2.G and the packaging plasmid pCMV-dR8.91 were obtained via Addgene (www.addgene.org), whereas the lentiviral vectors pLVCT-tTRKRAB-dsRed and pLV-T-EGFP were kindly provided by prof. D. Schümperli (University of Bern). The human B^0AT1 and TMEM27 coding sequences were subcloned in pLV-T-EGFP in place of EGFP sequence. Bulk production of the different plasmids and vectors was performed transforming HB101 *E.coli* cells with the constructs, followed by midi-prep DNA extraction kit (Qiagen).

HEK293T (below passage 20) were cultured according to the cell culture protocol. For transfection procedure, HEK293T were counted and $4\text{-}5\cdot10^6$ cells were seeded on 10 cm dishes and allowed to attach overnight. The following day, medium change was performed two hours before transfection. The transfection mixture was prepared as follows: 5 µg of pMD2.G, 10 µg of pCMV-pR8.91 and 15 µg of the vector of interest. Volume was adjusted to 450 µL with 0.1x TE buffer pH 8.8. 50 µL of 2.5 M $CaCl_2$ were added dropwise to the DNA mixture and the volume was adjusted to 500 µL with 2x HBS, mixed well and let complex for at least 20 min at RT. DNA transfection mixture was added dropwise to the cell culture dish and medium was exchanged the next morning. The supernatant was collected from transfected HEK293T cells 48 hours post transfection, filtered through 0.45 µm filters and centrifuged at 25000 rpm (Sorvall® Ultraspeed Centrifuge/Rotor TH64I) for 90 min at 4°C. Supernatant was aspirated almost completely and the invisible pellet was resuspended in approx. 1 mL of the same medium. The same day of virus harvesting, MDCK cells were trypsinized, counted and $1\cdot10^6$ cells were resuspended with the virus containing medium and plated on a 35 mm petri dish. After overnight incubation, the medium was replaced with MDCK standard medium and the cells were allowed to reach confluency.

MDCK cells inducibly overexpressing B^0AT1 and/or TMEM27 were obtained as shown in Figure 11. After the third transduction, cells were cloned by limiting dilution. Aliquots of cell suspension corresponding to 0.5, 1 or 2 cells were seeded in 96-well plates and monitored for two weeks. Cell populations deriving from one

single cell were selected and tested for the inducible and stable expression of B^{0}AT1 and/or TMEM27 by RT-PCR and Western blotting.

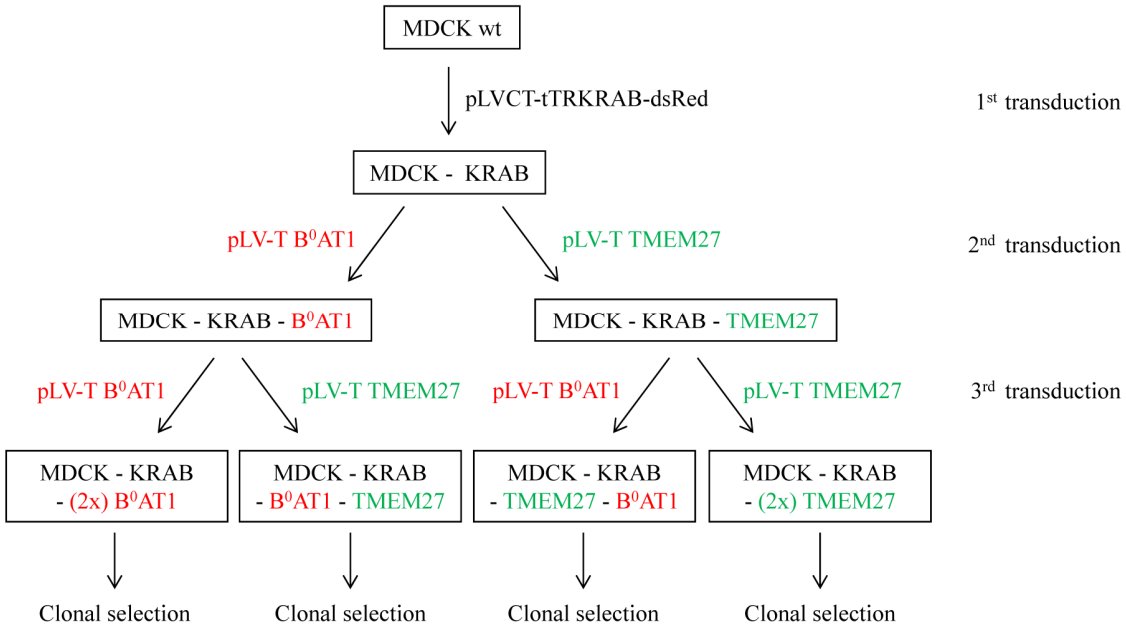

Figure 11. Sequential cotransduction of MDCK cells with KRAB, B^{0}AT1 and TMEM27 constructs. MDCK-KRAB cells were first transduced either with B^{0}AT1 or with TMEM27 constructs. Obtained cell lines were secondly transduced with B^{0}AT1 or with TMEM27 construct resulting in either double transduced B^{0}AT1 or double transduced TMEM27 or B^{0}AT1-TMEM27 co-expressing cell lines.

4.1.3 Western Blotting

MDCK cells grown on plastic supports (6-well plates or 10 cm dishes) or polycarbonate filters (24 mm filters) were washed once with ice-cold PBS, scraped from their support in 500-1000 µL of ice-cold PBS and centrifuged at 2000 g for 2 min at 4°C. Cell pellet was then homogenized by vigorous pipetting in 20-200 µL of ice-cold Radio-Immunoprecipitation Assay (RIPA) buffer (150 mM NaCl, 50 mM Tris pH 7.4, 1% NP-40, 0.5% Na-Deoxycholate) supplemented with fresh Protease Inhibitor Cocktail (Sigma-Aldrich) and incubated for 40 min on ice. Cell debris was pelleted with centrifugation at 2500 g for 10 min at 4°C and the supernatant was either used immediately for protein quantification or snap frozen and stored at -80°C. Protein sample concentration was determined by DC-Protein Assay,

following the manufacturer's guidelines (Bio-Rad) and using the BioTek Microplate Spectrophotometer μQuant.

For protein deglycosylation assay, 20 μg of protein lysate were treated with N-Glycosidase F (PNGase F) according to the manufacturer's guidelines (New England Biolabs). The samples were denatured at 65°C for 15 min in the provided buffer containing β-Mercaptoethanol and SDS, incubated for 1 h at 37°C in reaction buffer with 1% NP-40 with or without added PNGase F.

For Western blotting experiments, 20 μg of protein lysate were diluted in 4x Laemmli buffer supplemented with 10% β-Mercaptoethanol, incubated at 37°C for 30 min and loaded on 10% polyacrilamide gel. After separation by electrophoresis, proteins were transferred to PVDF membrane (Immobilion-P, Millipore). Blots were blocked (1 h at RT) with 5% milk powder in Tris-buffered saline supplemented with 0.1% Tween-20 (TBS-T), and incubated (overnight at 4°C) with the primary antibody diluted in 5% milk in TBS-T as described in Table 2. After washing the blot with TBS-T, secondary antibody diluted in 5% milk in TBS-T was applied (1 h at RT) and the antibody binding was detected with Immobilion Western Chemiluminescent HRP substrate (Millipore) or CDP-Star (Roche) and visualized with FujiFilm Las-4000 camera according to the manufacturer's instructions. Image-J software was used for densitometric analysis of the Western blots.

Antibody	Host	Dilution	Source	Catalogue #
B^0AT1	Rabbit	1:1000	In house	
TMEM27	Mouse	1:1000	Abnova	H00057393-B01P
β-Actin	Mouse	1:5000	Sigma-Aldrich	A5316
Anti-rabbit, HRP conjugate	Goat	1:5000	Promega	W401B
Anti-mouse, HRP conjugate	Goat	1:5000	Promega	W402B
Anti-rabbit, AP conjugate	Goat	1:5000	Promega	S373B
Anti-mouse, AP conjugate	Goat	1:5000	Promega	S372B

Table 2. List of primary and secondary antibodies used in Western blot experiments.

4.1.4 RNA extraction and RT-PCR

MDCK cells were grown on plastic dishes and B^0AT1 and/or TMEM27 expression was induced for 3 days with 1 µg/mL doxycycline. Cells were washed once with ice-cold PBS, scraped from their support in 500-1000 µL of ice-cold PBS and centrifuged at 2000 g for 2 min at 4°C. RNA was isolated using RNeasy Mini Kit (Qiagen), following the manufacturer's instructions. Cells-to-cDNA II kit (Applied Biosystems) was used to isolate RNA from MDCK clonal cell lines in the initial screening test. RNA concentration was measured by NanoDrop ND 1000 (NanoDrop Technologies).

Reverse transcription of the isolated RNA was performed with TaqMan® RT Kit using random hexamers (Applied Biosystems).

Validation of exogenous B^0AT1 and TMEM27 insertion into MDCK genome of clonal cells was performed by PCR, using primers designed for the human genes and listed in Table 3. Similarly, endogenous expression of TMEM27 and ACE2 was tested by PCR, using primers designed for the canine genes and listed in Table 3. In both experiments, the reaction mix consisted of 1x reaction buffer, 5 mM $MgCl_2$, 400 µM dNTPs, 0.1 µM of each primer, 1 U of Taq Polymerase (EurobioTaq) and water adjusted to a final volume of 20 µL. 5 ng of cDNA were used per reaction.

Target mRNA	*Specie*	*Primer sequence (5'→3')*
HPRT	Mouse	Sense: TTATCAGACTGAAGAGCTACTGTAATGATC Antisense: TTACCAGTGTCAATTATATCTTCAACAATC
B^0AT1	Human	Sense: GTGTGGACAGGTTCAATAAGGACAT Antisense: CCACGTGACTTGCCAGAAGAT
TMEM27	Human	Sense: CCTCTTCAAAGCGATGGTAGCT Antisense: CCCTCTGGGTTACATTGCAAA
TMEM27	Dog	Sense: TCAGCCAGATGCAGAGAATG Antisense: CTCATGGAGAAAGCCACCAT
ACE2	Dog	Sense: AGCCCAACTGGATGCCTCCCA Antisense: TGCATCCCAGCTCTGGTTCACCA

Table 3. List of primers used for PCR analysis.

The following PCR program was used:

Temperature	Time	Cycles
94°C	2 min	
94°C 60°C 72°C	30 sec 30 sec 30 sec	35
72°C	5 min	

Finally, PCR amplification products were separated on a 2% agarose gel electrophoresis and analyzed with the Red™ imaging system (Alpha Innotech).

4.1.5 Immunofluorescence staining

MDCK cells were grown on poly-D-lysine (Sigma-Aldrich) coated coverslips or differentiated on polycarbonate filters according to the cell culture protocol. B^0AT1 and/or TMEM27 expression was induced for 3 days (coverslips) or 2 days prior the seeding and the whole length of differentiation (filters) with 1 µg/mL doxycycline. Cells were washed once with ice-cold PBS and fixed with methanol-acetone (1:1) for 5 min at -20°C. Cells were washed repetitively with ice-cold PBS and incubated with PBS-Triton X-100/BSA (0.1%/2%) for 30 min at RT. The same blocking solution was used to incubate primary and secondary antibodies. Incubation with primary antibody was performed in a humidified chamber overnight at 4°C. After washing, cells were incubated for 1 h at RT with secondary antibodies. 4',6-Diamidino-2-Phenylindole Dihydrochloride (DAPI, Life Technologies) was added to the secondary antibody mixture (1:5000 dilution) in order to counterstain the nuclei. Coverslips or filter pieces were mounted in DAKO-Glycergel and analyzed at the fluorescent light microscope (Nikon) or confocal laser scan microscope (Leica).

Antibody	Host	Dilution	Source	Catalogue #
B^0AT1	Rabbit	1:1000	In house	
TMEM27	Mouse	1:1000	Abnova	H00057393-B01P
Anti-rabbit, Alexa Fluor 594	Donkey	1:2000	Life Technologies	A21207
Anti-mouse, Alexa Fluor 488	Donkey	1:2000	Life Technologies	A21202

Table 4. List of primary and secondary antibodies used in immunofluorescence staining experiments.

4.1.6 Amino acid uptake

MDCK cells were grown on filters according to the standard cell culture protocol. B^0AT1 and/or TMEM27 expression was induced 2 days prior the seeding and then for the all length of differentiation with 1 µg/mL doxycycline. The trans-epithelial electrical resistance across intact monolayers of MDCK cells was measured using EVOHM device (World Precision Instruments). Cells were washed three times and then incubated for 30 min at 37°C with uptake buffer (150 mM NaCl, 10 mM HEPES pH 7.4, 1 mM $CaCl_2$, 5 mM KCl, 1 mM $MgCl_2$, 10 mM glucose). Fresh uptake buffer was then applied on the basolateral side whereas the apical compartment received the uptake buffer supplemented with 1 mM L-Leucine and the corresponding ^{3}H-labeled L-Leucine (Harmann Analytic) as tracer. ^{14}C-labeled mannitol (Hartmann Analytic) was used as a control for the integrity of the cell monolayer. After 10 min incubation at 37°C, the uptake was stopped by replacing the apical and basolateral solutions with ice-cold uptake buffer. The cells were washed three times and the filters were excised and placed into scintillation fluid and shaked overnight at RT. Radioactivity was measured by liquid scintillation analyzer (Packard Tri-Carb 2900TR, PerkinElmer).

4.1.7 Immunoprecipitation

MDCK cells were grown on 10 cm dishes and B^0AT1 and/or TMEM27 expression was induced for 3 days with 1 µg/mL doxycycline. Cell lysates were prepared according to the section 4.1.3. 500 mg of protein lysate were preincubated with primary antibodies for 3 hours at 4°C under gentle rocking. Lysates were then added to the 50 µL of protein A/G conjugated agarose beads slurry (Thermo Scientific) and incubated overnight at 4°C under gentle rocking. Beads were washed

three times in 50 mM NaCl, 50 mM HEPES pH 7.4, 5 mM EDTA, 1% NP-40 and elution of the bound proteins was performed by incubation with Laemmli buffer (with β-Mercaptoethanol) at 60°C for 40 min. Protein samples were then subjected to Western blot analysis.

4.1.8 Pulse-chase analysis

MDCK cells were grown on 60 mm dishes and B^0AT1 and/or TMEM27 expression was induced for 3 days with 1 µg/mL doxycycline. Cells were washed three times with methionine-free DMEM (Sigma-Aldrich) supplemented with cysteine and 10% FBS and then incubated in the same medium for 30 min 37°C, 5% CO_2. The medium was exchanged for the above-mentioned medium containing 125 µCi/mL of ^{35}S-Methionine for 1 h at 37°C, 5% CO_2. For chase experiments, cells were subsequently incubated in complete culture medium for the times indicated. Cells were then washed three times in ice-cold PBS containing Ca^{2+} and Mg^{2+} and cell lysates were prepared accordingly to the section 4.1.3. Incorporated radioactivity was determined by TCA precipitation and liquid scintillation.

4.1.9 Statistics

Analysis of the experimental data was performed by Graph Pad Prism and MS Office-Excel. Differences between mean values of groups were tested with one-way analysis-of-variance (ANOVA) followed by Tukey's post-test. Level of significance are given as * = p≤0.05, ** = p≤0.01, *** = p≤0.001. Data are expressed as mean ± SEM.

4.2 Results

4.2.1 Test of anti-B^0AT1 and anti-TMEM27 antibodies

In order to study the function, localization and regulation of B^0AT1 protein, polyclonal antibodies were raised against a C-terminal peptide of human B^0AT1 by Pineda, Berlin. The rabbit antiserum was then affinity purified and antibody specificity was tested in Western blot experiments with lysates from HEK293T cells transiently transfected with B^0AT1 (Fig. 12, Panel A). Two specific bands presumably corresponding to fully- and core-glycosylated B^0AT1 were detected at 100 kDa and 72 kDa, respectively. Treatment with PNGase F showed a double band around 55 kDa corresponding to the deglycosylated B^0AT1. In addition, a smeared band was observed around 46 kDa. Heating of the sample and addition of PNGase F buffer caused a signal reduction of the higher molecular weight bands and the disappearance of the low molecular weight band which likely corresponds to a proteolytically cleaved form of B^0AT1.

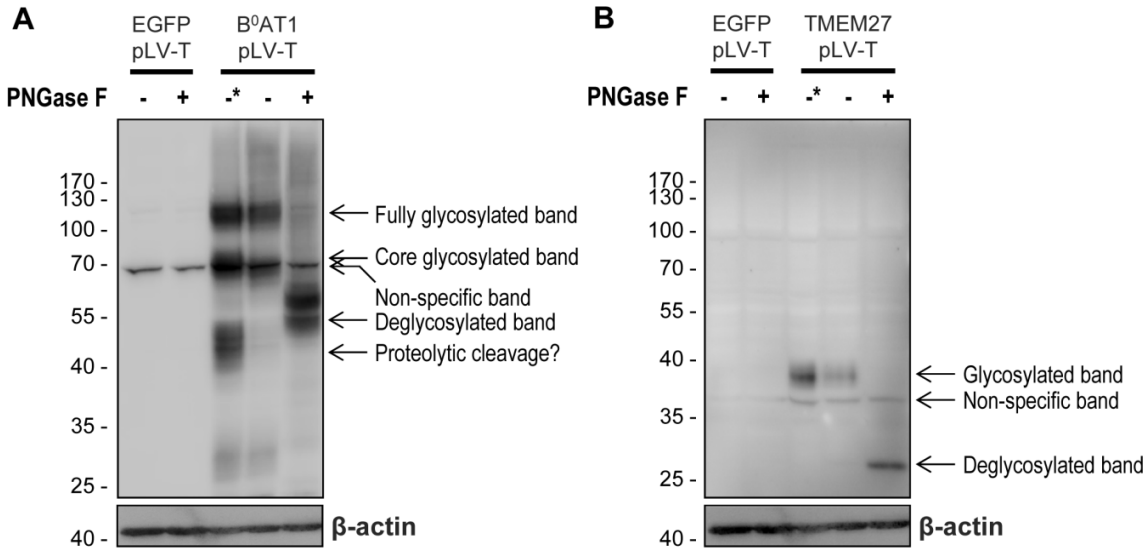

Figure 12. Characterization of anti-B^0AT1 and anti-TMEM27 antibodies. Cell lysates from HEK293T cells transfected with B^0AT1 or TMEM27 were resolved with SDS-PAGE after treatment with (+) or without (-) Peptide-*N*-glycosidase F (PNGase F) enzyme. After Western blot protein transfer membranes were incubated with anti-B^0AT1 (panel A) or anti-TMEM27 (panel B) antibodies. β-actin was used as loading control. * indicates samples which were neither heated nor incubated with PNGase F buffer.

Next, we tested commercial polyclonal antibodies raised in mouse against the full length of human TMEM27 sequence. Lysates from HEK293T cells transiently transfected with TMEM27 showed a specific signal in Western blot experiments when compared to the control lane (Fig. 12, panel B). In addition, PNGase F treatment showed a single band at ~27 kDa corresponding to the deglycosylated protein.

4.2.2 Overexpression of B^{0}AT1 and TMEM27 in MDCK cells using a lentiviral Tet-On system

To better understand the interaction between B^{0}AT1 and TMEM27, as well as the impact of the renal accessory protein on B^{0}AT1 expression and stability, MDCK cells were chosen as *in vitro* model. Unfortunately, constitutive overexpression of B^{0}AT1 and TMEM27 in MDCK cells was not tolerated by the cells. Transduced MDCK cells either lost their phenotype, not forming tight epithelia anymore, or the expression of the amino acid transporter and its accessory protein was rapidly lost within few passages (Lisa Ruckstuhl, Lisa Arps – unpublished data). To overcome this problem we used a KRAB repressor based inducible system. Doxycycline treatment demonstrated a significant induction of B^{0}AT1 and TMEM27 protein expression after the first transduction, as shown by Western blot (Fig. 13, panel A) and immunofluorescence microscopy (Fig. 13, panel B) experiments.

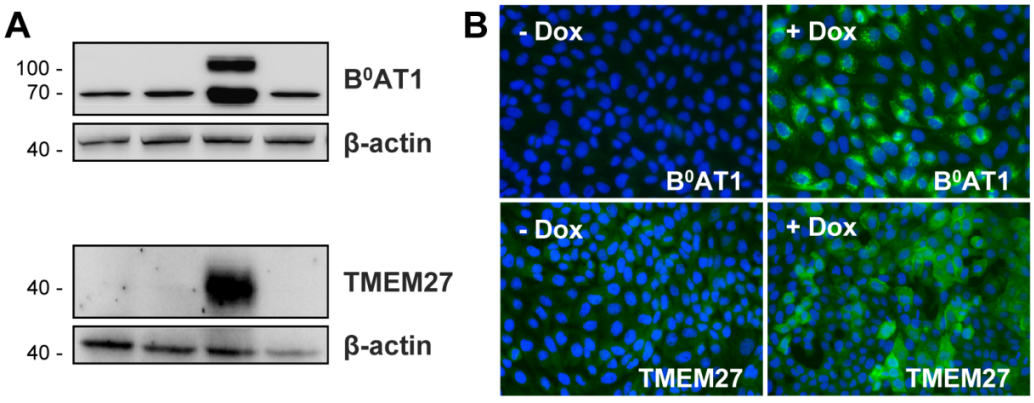

Figure 13. Inducible overexpression of B^{0}AT1 and TMEM27 in MDCK cells. MDCK cells transduced with B^{0}AT1 or TMEM27 were grown on plastic or on poly-D-lysine coated cover-slips in presence (+) or absence (-) of doxycycline. Western blot (panel A) and immunofluorescence microscopy (panel B) showed a significant induction of B^{0}AT1 and TMEM27 protein in transduced cells.

Induction with doxycycline resulted in co-expression and co-localization of B^{0}AT1 and TMEM27 proteins in B^{0}AT1-TMEM27 transduced MDCK cells, as detected by immunofluorescence microscopy (Fig. 14).

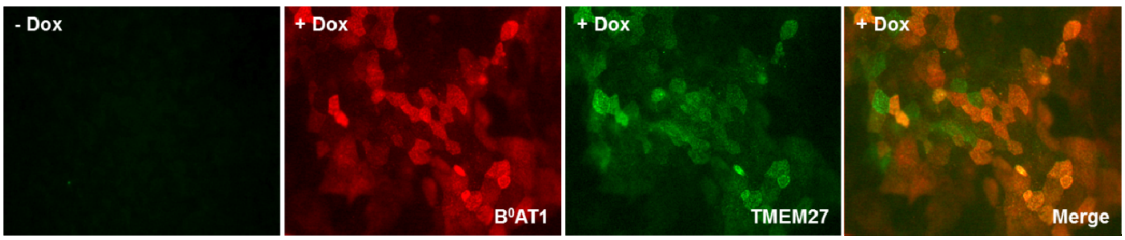

Figure 14. Co-expression of B^{0}AT1 and TMEM27 in MDCK cells. B^{0}AT1-TMEM27 overexpressing MDCK cells were grown on poly-D-lysine coated cover-slips in presence (+) or absence (-) of doxycycline. Immunodetection of B^{0}AT1 (red) and TMEM27 (green) in double transduced MDCK cells showed co-expression of both protein and a partial co-localization (orange).

Since the obtained mixed populations showed a non homogenous expression of both gene products, we decided to generate clonal cell lines with the expectation that they would display a more uniform transgene expression. Therefore, the above described mixed population MDCK cell lines were subjected to a limited dilution. Six clones per each cell line were selected based on the mRNA expression of the transduced gene (data not shown). A second screening based on protein expression resulted in a reduced number of working clones. Three clones of each cell line expressing the protein alone and three clones of the cell line co-expressing the two proteins together were selected. Western blot results demonstrated that clonal cell lines have differences in level of B^{0}AT1 and TMEM27 protein expression (Fig. 15, panel A). Unlike expected, immunofluorescence data showed that clonal cell lines co-expressing B^{0}AT1 and TMEM27 do not present a uniform expression pattern of both gene products. In fact, individual cells express either B^{0}AT1 or TMEM27 and only few cells co-express both proteins (Fig. 15, panel B).

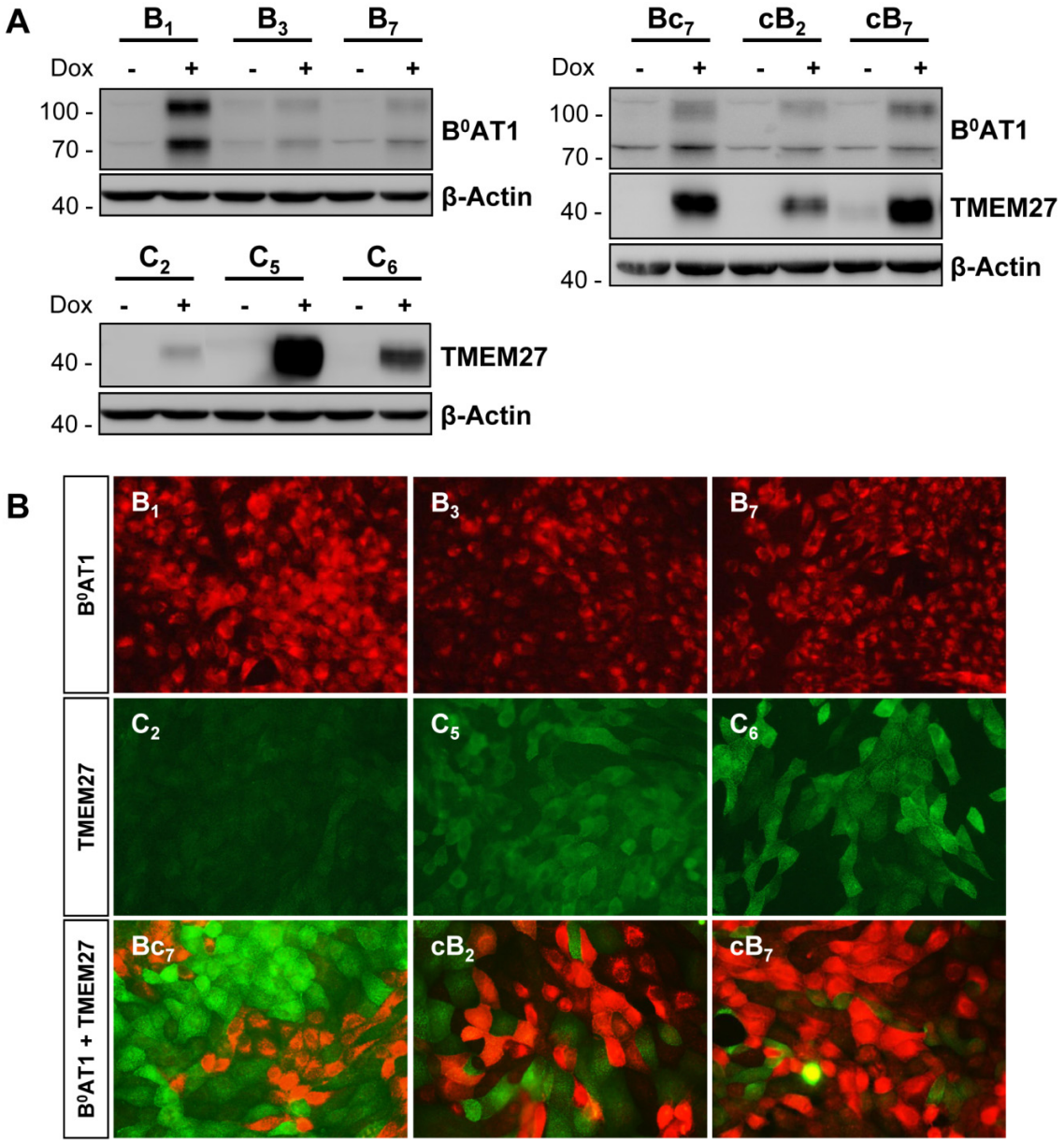

Figure 15. Protein-based screening of B^{0}AT1 and/or TMEM27 transduced MDCK clonal cell lines. B^{0}AT1 and/or TMEM27 overexpressing MDCK clones were grown on plastic or on poly-D-lysine coated cover-slips in presence (+) or absence (-) of doxycycline. Western blot (panel A) and immunofluorescence microscopy (panel B) showed different levels of B^{0}AT1 and TMEM27 protein expression among the clones. Immunodetection of B^{0}AT1 is shown in red, TMEM27 in green, whereas orange indicates co-localization.

4.2.3 Effect of TMEM27 on B^0AT1 function

To test if TMEM27 may impact on B^0AT1 transport activity, functional studies were performed. Leucine transport was measured in MDCK cells grown on polycarbonate filters. Trans-epithelial resistance (TEER) of the monolayer was measured before the uptake experiment and MDCK clones formed a tight epithelium (TEER ≥ 1 kΩ·cm^2) when grown on permeable supports. In addition, we observed significantly higher Na^+-dependent uptake of B^0AT1 substrate amino acid in cells co-expressing B^0AT1 and TMEM27 compared to the wild-type cells or cells expressing B^0AT1 alone (Fig. 16).

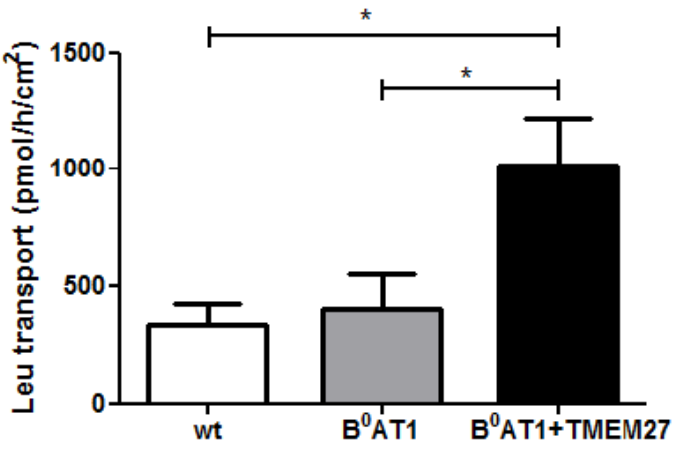

Figure 16. TMEM27 upregulates B^0AT1 function in MDCK cells. Wild-type MDCK cells or clonal cell lines transduced with either B^0AT1 or B^0AT1 and TMEM27 were grown on permeable supports. The apical uptake of 1 mM L-leucine was measured for 10 min in presence of Na^+. Data are represented as mean ± SEM of 3 biological replicates; * = p≤ 0.05, one-way ANOVA followed by Tukey's post-test.

4.2.4 Effect of TMEM27 on B^0AT1 localization

To understand whether the effect of TMEM27 on B^0AT1 transport is caused by changes of B^0AT1 surface expression, confocal immunofluorescence microscopy was performed on MDCK cells grown on filters. In absence of TMEM27, B^0AT1 showed predominantly cytosolic expression (Fig. 17, upper panel, left picture). In contrast, cells co-expressing B^0AT1 and TMEM27 showed surface expression of both proteins (Fig. 17, lower panel). However few cells expressing B^0AT1 alone showed surface localization of the amino acid transporter (Fig. 17, upper panel, right

picture). We hypothesized that the endogenous TMEM27 or ACE2 might play a role in the surface localization of B^{0}AT1 in absence of the exogenous TMEM27. We therefore analyzed the endogenous expression of the two accessory proteins of B^{0}AT1 by RT-PCR. Both TMEM27 and ACE2 canine mRNA expression could be detected in MDCK cells (Fig. 18).

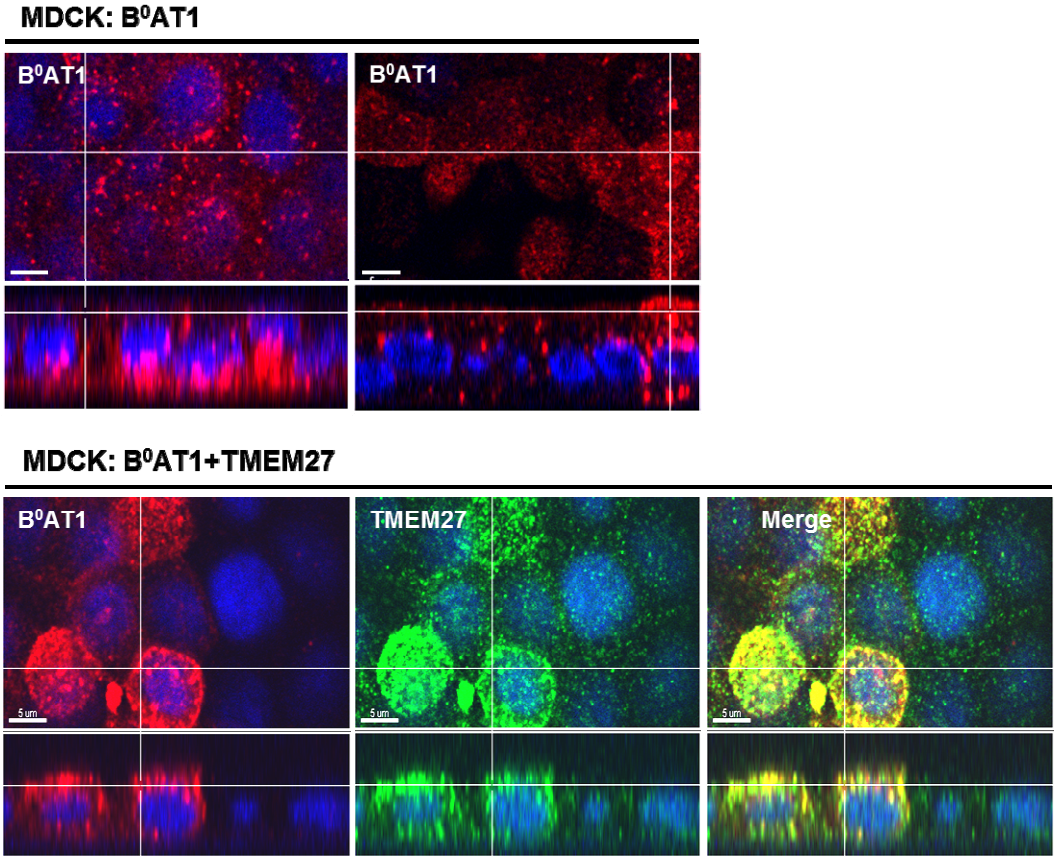

Figure 17. TMEM27 changes B^{0}AT1 localization in MDCK cells. MDCK cells were grown on polycarbonate filters. Immunofluorescence staining followed by laser confocal scanning microscopy was performed on clonal MDCK cell lines expressing either only B^{0}AT1 (upper panel) or co-expressing B^{0}AT1 and TMEM27 (lower panel). Co-localization of B^{0}AT1 and TMEM27 is shown in yellow.

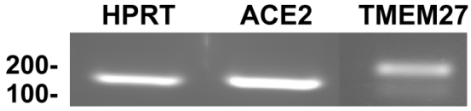

Figure 18. MDCK cells endogenously express ACE2 and TMEM27 at the mRNA level. Reverse transcription PCR was performed with material from B^{0}AT1 expressing MDCK cells. Data showed ACE2 and TMEM27 mRNA expression in MDCK cells. HPRT was used as control.

4.2.5 Does TMEM27 interact with B^{0}AT1 *in vitro*?

TMEM27 has previously been shown to interact with B^{0}AT1 *in vivo* (Danilczyk et al., 2006). To test the role of their interaction *in vitro* MDCK cells co-expressing B^{0}AT1 and TMEM27 were used. Immunoprecipitation with anti-B^{0}AT1 antibody followed by Western blotting using the same antibody demonstrated a successful immunoprecipitation of B^{0}AT1 protein (Fig. 19). However, a low increase in B^{0}AT1 signal upon immunoprecipitation compared to the total abundance was observed (Fig. 19, see IP fraction versus total input in B^{0}AT1 Western blot). In addition, anti-TMEM27 antibody did not confirm the physical interaction *in vitro.* Under none of the tested conditions (high salt vs low salt buffer, more or less stringent detergents) co-immunoprecipitation of TMEM27 with B^{0}AT1 antibodies was possible (data not shown).

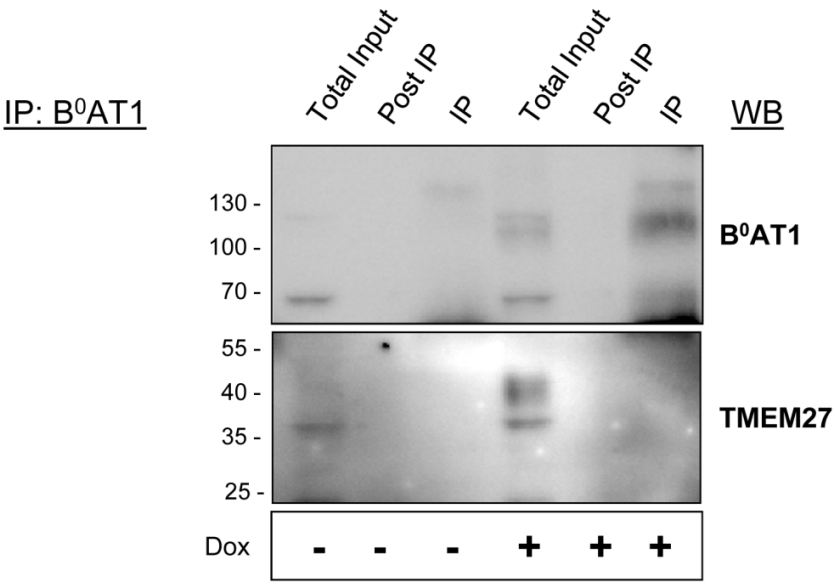

Figure 19. TMEM27 it is not quantitatively co-immunoprecipitated with B^{0}AT1 in MDCK cells. Clonal MDCK cells overexpressing B^{0}AT1 and/or TMEM27 were grown on plastic in presence (+) or absence (-) of doxycycline. Cell lysates were incubated overnight with anti-B^{0}AT1 antibodies. Immunoprecipitated complexes were analyzed by Western blotting using anti-B^{0}AT1 and anti-TMEM27 antibodies. **Post-IP**: not bound fraction; **IP:** immunoprecipitated fraction.

4.2.6 Effect of TMEM27 on B^{0}AT1 protein stability

To investigate the impact of TMEM27 co-expression on B^{0}AT1 protein stability, we performed pulse-chase analysis followed by immunoprecipitation with anti-B^{0}AT1 antibody. Shortly after pulse, the core-glycosylated band of B^{0}AT1 was detected at ~66 kDa in both, presence or absence of TMEM27 (Fig. 20). Importantly, during the chase period, B^{0}AT1 was observed maturing to the fully glycosylated form of ~100 kDa. The fully glycosylated protein band was not clearly stabilized by TMEM27 co-expression. In addition, co-precipitation of TMEM27 with anti- B^{0}AT1 antibody was not successful (data not shown).

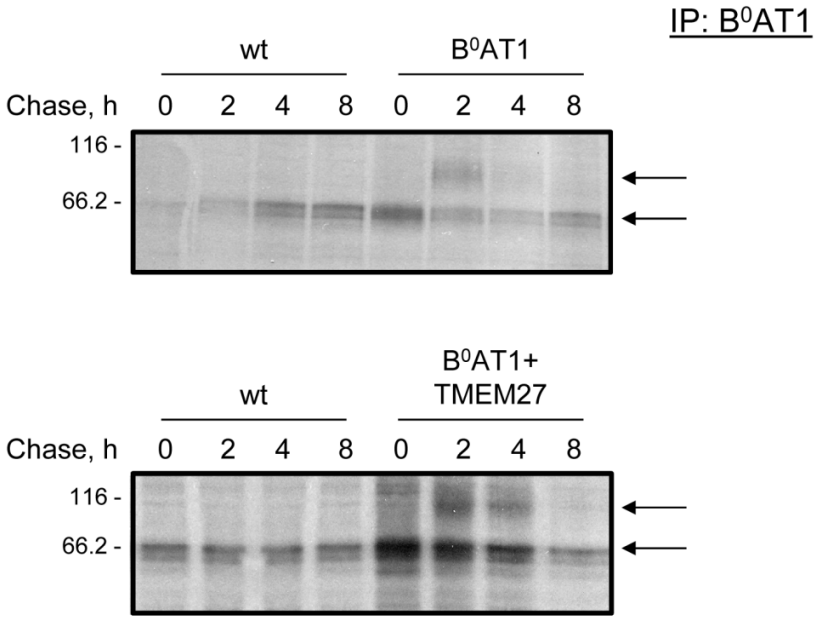

Figure 20. TMEM27 does not stabilize B^{0}AT1 in MDCK cells. B^{0}AT1 immunoprecipitation was performed on lysates of wild type (wt), B^{0}AT1 or B^{0}AT1 and TMEM27 overexpressing MDCK cell clones that were pulse-labelled for 1 h and chased for the number of hours indicated. Lower arrow indicates core-glycosylated B^{0}AT1 whereas the upper one shows the fully-glycosylated B^{0}AT1.

Taken together, the data suggested that TMEM27 increases B^{0}AT1 function by upregulating its surface expression. The physical interaction between the two proteins shown *in vivo* was not proven *in vitro*. Furthermore, it remains unclear whether TMEM27 regulates B^{0}AT1 protein stability.

4.3 Discussion

B^0AT1 is the main neutral amino acid transporter expressed on the apical membrane of renal proximal tubules and intestinal epithelial cells (Romeo et al., 2006). Renal expression and function of B^0AT1 have been shown to depend on the co-expression of TMEM27 (Danilczyk et al., 2006). The initial goal of this thesis was to characterize the cellular and molecular mechanisms underlying the interaction between B^0AT1 and TMEM27 *in vitro*. B^0AT1-TMEM27 co-expression in MDCK cells confirmed the importance of TMEM27 for B^0AT1 function. Also, we provided conclusive evidence that TMEM27 is an important regulator of B^0AT1 trafficking, as TMEM27 expression is required for B^0AT1 correct cell surface localization.

4.3.1 B^0AT1-TMEM27 overexpression in MDCK cells is inducible but not uniform

We generated MDCK cell lines overexpressing B^0AT1 and/or TMEM27 under the control of an inducible promoter. This cell line of distal nephron origin represents a well-established model for epithelial polarity studies (Misfeldt et al., 1976; Cereijido et al., 1978). The choice of a conditional lentiviral system over the classical constitutive expression system was due to the hypothesis that the luminal neutral amino acid transporter overexpression could be toxic for the cells. In fact, several attempts performed by former students in our group resulted in changes in MDCK cell morphology and loss of B^0AT1 expression along the cell culture passages (unpublished data). Mixed populations of MDCK cells transduced with KRAB-B^0AT1-TMEM27 showed a normal morphology and a considerable induction of the amino acid transporter and its accessory protein upon treatment with doxycycline. To obtain a more homogenous cell population, we isolated MDCK clonal cells which exhibited high levels of B^0AT1-TMEM27 protein expression. In contrast to our expectations, B^0AT1-TMEM27 overexpressing clonal cell lines did not show a uniform expression pattern of B^0AT1 and TMEM27 proteins. More precisely, the gene product transduced in the first place was more expressed than the second one. Previous studies indicated that the transduction efficiency of a retroviral vector is dramatically decreased if the cells have been already transduced with another

retroviral vector (Walker et al., 1996). Thus, the viral interference could explain the weak co-expression of both transgenes in our MDCK cell model.

4.3.2 TMEM27 increases B^0AT1 function by upregulating its surface expression

Several amino acid transporters require accessory proteins for their correct localization on the plasma membrane and function. The best characterized examples are the heavy chains and the heterodimeric amino acid transporters (SLC3 and SLC7 family). The heavy chain rBAT (SLC3A1) interacts with the catalytic light chain $b^{0,+}$AT (SLC7A9) to which it is linked by a disulphide bridge. Interestingly, rBAT has been shown to be necessary for the correct trafficking of the complex to the plasma membrane (Pfeiffer et al., 1999a). Likewise, CD98/4F2hc forms a physical complex with several SLC7 family members (namely LAT1, LAT2, y^+LAT1, y^+LAT2, xCT, ASC1 (SLC7A5, 8, 7, 6, 11 and 10, respectively)) and is required for their correct localization on the basolateral membrane of renal proximal tubular cells. However, a few other partner proteins have been suggested to have a negative interaction with amino acid transporters. For example, the Glutamate Transport Associated Protein for EAAT3 (SLC1A1) (GTRAP3-18) is known to downregulate EAAT3-mediated glutamate transport by delaying EAAT3 traffic from the ER to the Golgi (Ruggiero et al., 2008). Here, we showed that TMEM27 increases B^0AT1 function by upregulating its surface expression. In the absence of TMEM27, the heterologous B^0AT1 was still expressed in MDCK cells but did not correctly traffic to the apical membrane of epithelial cells. However, few cells expressing B^0AT1 in the absence of TMEM27 showed an apical localization of the amino acid transporter. We hypothesized that the canine TMEM27 and ACE2, shown to be expressed in MDCK cells at the mRNA level, might allow the delivery of B^0AT1 to the luminal membrane in the absence of exogenous TMEM27. Taken together, our data confirmed that B^0AT1 requires TMEM27 for a correct apical localization.

The cellular and molecular mechanisms underlying B^0AT1 and TMEM27 interaction are still unclear. In contrast to the published *in vivo* data (Danilczyk et al., 2006), the present immunoprecipitation experiments did not show a direct binding

between B^0AT1 and TMEM27 *in vitro*. The reason for this differential response is uncertain. The experimental conditions (ionic strength of the buffer, detergents, etc.) might have been not optimal for the co-immunoprecipitation, the sensitivity of the system not sufficient to reveal it or an additional partner protein stabilizing the interaction *in vivo* missing. The association between B^0AT1 and TMEM27 has been already shown to occur through non-covalent interactions, as both proteins migrate with the same mobility, both under reducing and non-reducing conditions (Danilczyk et al., 2006). The difficulties encountered in co-immunoprecipitation of B^0AT1 with TMEM27 suggest a low affinity or transient interaction between the two proteins. Interestingly, TMEM27 has been shown to bind to the N-ethylmaleimide-sensitive fusion attachment receptor (SNARE) complex proteins by directly interacting with the vesicular protein snapin in pancreatic β-cells (Fukui et al., 2005) and in renal collecting duct cells (Zhang et al., 2007; Yasuhara et al., 2008). This interaction has been suggested to be crucial for the exocytosis of insulin (Fukui et al., 2005) and vesicular trafficking of polycystin-2 (Zhang et al., 2007). Nevertheless, there is no clear evidence, with the exception of EAAC1 (Fournier and Robinson, 2006), that amino acid transporters trafficking is mediated by SNARE proteins.

It is still unclear whether B^0AT1 protein stability is affected by TMEM27 co-expression. *In vivo* data showed a dramatic decrease in B^0AT1 protein expression in brush border membrane vesicles of TMEM27 null mice (Danilczyk et al., 2006). Similarly, ACE2 knock-out mice displayed lack of B^0AT1 protein expression in the enterocytes (Camargo et al. 2009). These data suggested that B^0AT1 is rapidly degraded in absence of its accessory proteins. Previous studies indicated that when overexpressed in the absence of the light chain $b^{0,+}$AT, the heavy chain rBAT is rapidly degraded by the endoplasmic-reticulum-associated protein degradation (ERAD) pathway (Bartoccioni et al., 2008). In our study, pulse-chase analysis followed by immunoprecipitation with anti-B^0AT1 antibodies showed that both in the presence and absence of exogenous TMEM27 B^0AT1 is rapidly shifted to a terminally glycosylated form (Fig. 20). However, it is not possible to determine from our analysis whether this matured form of B^0AT1 is stabilized by the co-expression with TMEM27.

In summary, B^0AT1-TMEM27 inducibly overexpressing MDCK cells displayed normal epithelial phenotype and significant induction of both transgenes. However, this cell model showed some disadvantages, such as the uneven co-expression of the transgenes, the endogenous expression of TMEM27 and ACE2 and the loss of both transgenes expression along cell culture passages (this phenomenon has been further discussed in the manuscript). Due to these limitations, the interpretation of results was not always straightforward. To date, there is unfortunately not a good model for studying amino acid transporters in proximal tubule cells. Several cell lines deriving from various species such as human (HK-2), opossum (OK) or porcine (LLC-PK1) have been established. However, they exhibit de-differentiation markers since they lose the proximal tubule cell-specific biochemical and transport properties. Similarly, primary tubule cells present several disadvantages such as the limitation of the cell source, high variability and de-differentiation under *in vitro* conditions. In conclusion, although B^0AT1-TMEM27 overexpressing MDCK cells have a number of limitations, the ability to form polarized tight epithelia makes them a relevant cell model for the characterization of amino acid transporters trafficking and function. However, care must be taken when choosing the cell culture conditions and the overexpression system, as these parameters highly impact on the results.

5. MANUSCRIPT

Amino acids regulate transgene expression in MDCK cells

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My contribution to the manuscript concerns the study design and data interpretation under the supervision of F. Verrey. I performed all the experiments and wrote the paper with the help of F. Verrey.

Title: Amino acids regulate transgene expression in MDCK cells

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Abstract

Gene expression and cell growth rely on the intracellular concentration of amino acids that in metazoans depends on extracellular amino acid availability and transmembrane transport. To investigate the impact of extracellular amino acid concentrations on the expression of a concentrative amino acid transporter, we overexpressed the main kidney proximal tubule luminal neutral amino acid transporter B^0AT1-collectrin (SLC6A19-TMEM27) in MDCK cell epithelia. Exogenously expressed proteins co-localized at the luminal membrane and mediated neutral amino acid uptake. However, the transgenes were lost over few cell culture passages. In contrast, the expression of a control transgene remained stable. To test whether this loss was due to inappropriately high amino acid uptake, freshly transduced MDCK cell lines were cultivated either with physiological amounts of amino acids or with the high concentration found in standard cell culture media. Expression of exogenous transporters was unaffected by physiological amino acid concentration in the media. Interestingly, mycoplasma infection resulted in a significant increase in transgene expression and correlated with the rapid metabolism of L-arginine. However, L-arginine metabolites were shown to play no role in transgene expression. In contrast, activation of the GCN2 pathway revealed by an increase in eIF2α phosphorylation may trigger transgene derepression. Taken together, high extracellular amino acid concentration provided by cell culture media appears to inhibit the constitutive expression of concentrative amino acid transporters whereas L-arginine depletion by mycoplasma induces the expression of transgenes possibly via stimulation of the GCN2 pathway.

Introduction

Mammals have developed a finely tuned response to changes in nutrient availability. Amino acid sufficiency activates the mammalian target of rapamycin (mTOR) pathway, which ultimately promotes protein synthesis and cell growth. In contrast, amino acid limitation initiates an amino acid response (AAR) signaling cascade, which regulates multiple steps in gene expression including chromatin structure modification, transcription and translation [1]. Individual cells sense amino acid deficiency through an accumulation of uncharged tRNAs which bind to and activate the GCN2 protein kinase [2–4]. GCN2 protein kinase phosphorylates and inactivates the eukaryotic initiation factor 2α (eIF2α), which in turn leads to a decrease of global mRNA translation [4,5]. To compensate for this restriction, the expression of a spectrum of genes involved in the adaptive response to nutritional stress is stimulated. This phenomenon, known as translational derepression, was first described for the yeast transcription factor GCN4 [6], and then shown for the mammalian GCN4 homologue, activating transcription factor 4 (ATF4) [7]. ATF4 binds to C/EBP-ATF response elements (CARE)-containing genes and triggers their transcription. Among the genes induced by amino acid limitation, amino acid transporters (*SLC7A1*, *SLC38A2*), transcription factors (*CHOP*) or amino acid metabolic enzymes (*ASNS*) have been identified (reviewed in Ref. [1]).

The transport of amino acids across epithelial cells is mediated by a wide array of membrane proteins. Previously, to investigate the role of specific amino acid transporters, we reconstructed the transepithelial exchange of L-Arg and L-Leu by expressing the luminal heterodimeric amino acid antiporter $b^{0,+}$-rBAT (SLC7A9-SLC3A1) and the basolateral heterodimeric antiporters y^+LAT1-4F2hc (SLC7A7-SLC3A2) and LAT2-4F2hc (SLC7A8-SLC3A2) in MDCK epithelial cells [8,9]. This cell line of distal nephron origin represents a well-established model for epithelial polarity studies [10,11]. The overall directional (re)absorptive transport of amino acids is however driven by accumulative transporters

located in the luminal membrane of transporting epithelia. In the proximal kidney tubule and in the small intestine, this central role is mainly played by the luminal sodium symporter for neutral amino acids B^0AT1 (SLC6A19) [12,13]. Mutations in its gene have been shown to cause autosomal recessive Hartnup disorder [14,15]. The expression and function of B^0AT1 depends on the co-expression of members of the renin angiotensin system (RAS), namely TMEM27 (collectrin) in the kidney proximal tubule and angiotensin converting enzyme 2 (ACE2) in the small intestine [16,17]. Apart from dependence on organ-specific accessory proteins and recent data on the transcriptional control of its expression along the crypt-villus axis [18], the mechanisms controlling B^0AT1 expression and function are largely unknown.

Mycoplasma infection represents a well-known and insidious problem for cell culture users. Mycoplasmas are small bacteria (0.3-0.8 µm in diameter) which lack a cell wall and are not susceptible to many usual antibiotics. The major source of mycoplasma contamination in cell culture is the laboratory personnel, as the non-pathogenic mycoplasma (such as *M. orale*) colonize the oral cavity of humans and can easily spread to the laboratory equipment, media and reagents [19]. The effects of mycoplasma infections on the contaminated cell lines are variable and depend on the degree of infection. Previous studies have shown that mycoplasma use arginine as their major source of energy, converting it to ornithine and thereby producing adenosine triphosphate through a complex set of reactions [20]. The lack of an essential amino acid from the cell culture medium induces biochemical changes in the host-cell metabolism, which ultimately lead to a decrease in cell growth and cell death [21].

Since amino acid availability has been shown to affect the expression and activity of several amino acid transporters [22–24], the initial goal of the present study was to investigate the effect of extracellular amino acid availability on B^0AT1 expression, in particular at post-transcriptional levels. To construct an appropriate in vitro proximal tubule model, we co-expressed B^0AT1 with its renal accessory protein TMEM27 (B^0AT1-TMEM27) in MDCK cell epithelia under the control of the heterologous cytomegalovirus (CMV) promoter. Unlike for

the exogenous expression of antiporters previously mentioned, expression of B^0AT1-TMEM27 was unstable and inhibited by high extracellular amino acid concentrations. Furthermore, mycoplasma infection of MDCK cells increased expression of all tested exogenously expressed genes presumably via stimulation of the GCN2 pathway.

Materials and Methods

Cell culture

MDCK cells, kindly provided by Dr. N. Simmons (Newcastle University), were cultured at 37°C and 5% CO_2 in DMEM (catalog no. E15-810, GE Healthcare, Glattbrugg, Switzerland) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma-Aldrich, Buchs, Switzerland), 2 mM L-glutamine and 1% non essential amino acids (catalog no. M11-003, GE Healthcare).

Phoenix amphotropic retrovirus producer cells, kindly provided by Dr. G. Nolan (Stanford University), were cultured at 37°C and 5% CO_2 in DMEM (catalog no. E15-843, GE Healthcare) supplemented with 10% heat-inactivated FBS (Sigma-Aldrich), 2 mM L-glutamine and 1% non essential amino acids.

Human embryonic kidney cells (HEK293T), kindly provided by Dr. D. Schümperli (University of Bern) were grown in DMEM (catalog no. E15-810, GE Healthcare) supplemented with 10% heat-inactivated FBS (Sigma-Aldrich), 2 mM L-Glutamine and 1% non essential amino acids at standard cell culture conditions (37°C, 95% relative humidity and 5% CO_2).

Mycoplasma detection by PCR analysis

Mycoplasma infection in cell cultures was detected using Venor®GeM (Minerva biolabs, Berlin, Germany) following the manufacturer's instructions. 2 µL of medium deriving from confluent cell cultures were used for each PCR reaction.

Reagents

Physiological medium and arginine-free medium consisted of an amino acid-free DMEM (modified E15-810, custom-made GE Healthcare) supplemented with 5% FBS and physiological amino acid (1x PAA) concentrations or cell culture amino acid (except for arginine) concentrations, respectively, pH adjusted to 7.4, as listed in Table 1. Cell culture

tested amino acids, polyamines and urea (Sigma-Aldrich) were dissolved in Hanks' buffered salt solution (HBSS; GE Healthcare) in stock solutions of 10, 50 or 100 mM.

Cell treatments

Unless specified otherwise, MDCK cells (below passage 10) were seeded at confluent density ($1.7 \cdot 10^5$ cells/cm^2) and cultivated on Corning Costar Transwell filters (Corning, Amsterdam, The Netherlands) for 6 days in DMEM supplemented with 5% FBS, 2 mM L-glutamine and 1% non essential amino acids. In time course experiments with physiological levels of amino acids, cells were grown in regular DMEM and then treated for the last 1, 3 or 5 days of culture with 1x PAA medium. Cells were grown in regular DMEM for the first 3 days and then treated for 3 additional days with: *1)* amino acid-free medium supplemented with 0.5-8.0x PAA concentrations (dose response experiments); *2)* amino acid-free medium supplemented with the tested amino acids at 8x PAA levels and the remaining amino acids at 0.5x PAA concentrations (substrate specificity experiments). Cells were grown in arginine-free medium supplemented with 180 µM arginine for the first 3 days and then treated for 3 additional days with: *1)* arginine-free medium supplemented with 45, 180 or 720 µM arginine (arginine time course experiments); *2)* arginine-free medium supplemented with 720 µM arginine and NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME, 0-2000 µM; Sigma-Aldrich), arginase inhibitor Nw-Hydroxy-nor-L-arginine (nor-NOHA, 0-500 µM; Millipore, Zug, Switzerland) or α-difluoromethylornithine (DFMO, 0-3 mM; Sigma-Aldrich) (inhibitor experiments); *3)* arginine-free medium supplemented with 45 µM L-arginine in the presence or absence of 675 µM citrulline, ornithine, urea, D-arginine, or 10 µM putresceine, spermidine, spermine, or NO donor sodium nitroprusside (SNP, 0-10 µM; Sigma-Aldrich) (metabolite experiments); *4)* arginine-free medium supplemented with 720 µM arginine and HDAC inhibitor trichostatin A (TSA, 1 µM; Sigma-Aldrich).

cDNA constructs, transfection, and viral transduction

The human B^0AT1 cDNA sequence was inserted in the multiple cloning site of pIRES2-EGFP (catalog no. 6029-1, Life Technologies, Zug, Switzerland), upstream of the internal ribosomal entry site and the EGFP reporter gene. Human TMEM27 cDNA sequence was then subcloned in the above mentioned vector in place of EGFP sequence. The resulting bicistronic construct containing B^0AT1 and TMEM27 upstream and downstream of IRES, respectively, was then excised from the plasmid and inserted in the retroviral vector pLPCX (Clontech, Saint-Germain-en-Laye, France). EGFP was introduced in pLPCX as previously described [25]. Production of supernatants containing the pseudoviruses and subsequent transduction of MDCK target cells was performed as previously described [9]. The first subcultivation after transduction was defined as passage 1. Stable MDCK cell lines were selected and maintained in standard growth DMEM containing 2 µg/mL puromycin (Sigma-Aldrich). Human TMEM27 cDNA sequence was subcloned as a PCR fragment flanked by SmaI and XhoI restriction sites into the Eco47III and XhoI sites of pLenti6-EGFP (Life Technologies), thus yielding to pLenti6-TMEM27 vector. Lentiviral production was performed according to the protocol described elsewhere [26]. Infected MDCK cells were selected with 6 µg/mL blasticidin S (Life Technologies).

Antibodies

Polyclonal rabbit antibodies were raised against the synthetic peptide NH_2-NPGLDARIPSLAELEC-$CONH_2$ of human B^0AT1 and further affinity purified (Pineda, Berlin, Germany). Mouse anti-TMEM27 (Abnova, Taipei, Taiwan), mouse anti-EGFP (Clontech), rabbit anti-4E-BP1 (Cell Signaling, Danvers, MA, United States), rabbit anti-phospho-4E-BP1 (Thr70) (Cell Signaling), rabbit anti-eIF2α (Cell Signaling), rabbit anti-phospho-eIF2α (Ser51) (Cell Signaling), rabbit anti-ZO-1 (Life Technologies) and mouse anti-β-actin (Sigma-Aldrich) were used according to the manufacturers' instructions. Horse radish

peroxidase goat anti-rabbit IgG and alkaline phosphatase goat anti-mouse IgG secondary antibodies were purchased from Promega (Dübendorf, Switzerland).

Immunofluorescence staining of MDCK cells

MDCK cells on filters were washed twice with cold phosphate-buffered saline (PBS) supplemented with 1 mM $MgCl_2$ and 100 µM $CaCl_2$ (PBS^{++}) and fixed for 5 min in methanol:acetone (1:1) at -20°C. Filters were washed three times in PBS^{++} and nonspecific binding sites were blocked for 30 min at room temperature with 2% bovine serum albumin (BSA, Sigma Aldrich) in PBS^{++} supplemented with 0.1% Triton X-100 (Sigma Aldrich). Cells were incubated overnight with primary antibody to anti-B^0AT1, anti-TMEM27 or anti ZO-1 as indicated, diluted in 2% BSA in PBS^{++} supplemented with 0.1% Triton X-100. After washing, cells were incubated for 1 h at room temperature with Alexa Fluor 594 anti-rabbit-IgG antibody (Life Technologies) and Alexa Fluor 488 anti-mouse IgG antibody (Life Technologies). 4',6-Diamidino-2-Phenylindole Dihydrochloride (DAPI, Life Technologies) was added to the secondary antibody mix in order to counterstain the nuclei. Filter pieces were mounted in DAKO-Glycergel (Dako, Baar, Switzerland) and analyzed with a Leica TCS SP5 confocal laser scanning microscope (Leica, Heerbrugg, Switzerland) using a 63x objective lens (Leica). Digital images were processed using the software Imaris (Bitplane, Zurich, Switzerland),

Amino acid uptake

MDCK cells were grown on filters and the trans-epithelial electrical resistance across intact monolayers was measured using EVOHM device (World Precision Instruments, Sarasota, FL). Amino acid uptake was performed as previously described [9]. Briefly, cells were washed three times and then incubated for 30 min at 37°C with uptake buffer (150 mM NaCl, 10 mM HEPES pH 7.4, 1 mM $CaCl_2$, 5 mM KCl, 1 mM $MgCl_2$, 10 mM glucose). Fresh

uptake buffer was then applied on the basolateral side whereas the apical compartment received the uptake buffer supplemented with 1 mM L-Leucine and the corresponding ^{3}H-labeled L-Leucine (Hartmann Analytic, Braunschweig, Germany) as tracer. ^{14}C-labeled mannitol (Hartmann Analytic) was used as a control for the integrity of the cell monolayer. After 10 min incubation at 37°C, the uptake was stopped by replacing the apical and basolateral solutions with ice-cold uptake buffer. The cells were washed three times and the filters were excised and placed into scintillation fluid and shaked overnight at room temperature. Radioactivity was measured by liquid scintillation analyzer (Packard Tri-Carb 2900TR, PerkinElmer, Schwerzenbach, Switzerland).

Western blotting

Cells were lysed with ice-cold Radio-Immunoprecipitation Assay (RIPA) buffer (150 mM NaCl, 50 mM Tris pH 7.4, 1% NP-40, 0.5% Na-Deoxycholate) supplemented with fresh Protease Inhibitor Cocktail (Sigma) and PhosSTOP (Roche, Rotkreuz, Switzerland) and incubated for 40 min on ice. Cellular debris was pelleted with centrifugation at 2500 g for 10 min at 4°C and the protein concentration was determined by DC-Protein Assay, following the manufacturer's guidelines (Bio-Rad, Cressier, Switzerland). Cell extracts (20-40 µg protein) were resolved by SDS-PAGE on 10 or 12% gels and electrophoretically transferred to PVDF membranes (Immobilion-P, Millipore). Nonspecific binding sites were blocked for 1 h at room temperature with 5% powdered milk in Tris-buffered saline supplemented with 0.1% Tween-20 (TBS-T). Blots were then incubated overnight at 4°C with the primary antibody diluted in 5% powdered milk in TBS-T. After washing the blots with TBS-T, secondary antibody diluted in 5% powdered milk in TBS-T was applied for 1 h at room temperature and the antibody binding was detected with Immobilion Western Chemiluminescent HRP substrate (Millipore) or CDP-Star (Roche) and visualized with FujiFilm Las-4000 camera (GE-Healthcare) according to the manufacturer's instructions.

Image-J software (National Institutes of Health, Bethesda, MD) was used for densitometric analysis of the Western blots.

RT-PCR

Total RNA from cell pellet was isolated using RNeasy Mini Kit (Qiagen, Basel, Switzerland) following the manufacturer's instructions. Reverse transcription of the isolated RNA was performed with TaqMan® RT Kit using random hexamers (Life Technologies). Gene expression was quantified by quantitative real-time PCR using Taq Ready Mix™ (Life Technologies) as previously described [12]. Primers and probes are listed in Table 2. Probes were labelled with the reporter dye FAM at the 5' end and the quencher dye TAMRA at the 3' end (Microsynth, Balgach, Switzerland). β-Actin or 18S (Life Technologies) were used as housekeeping genes.

Amino acid measurements

Media were collected from MDCK cells grown on filters. Briefly, deproteinized samples were derivatized using EZ:faast™ kit (Phenomenex, Torrance, CA) and further analyzed by LC-MS/MS using API Sciex 2000 instrument (Ab Sciex, Brugg, Switzerland).

Cell count

Cell nuclei were visualized using 4',6-Diamidino-2-Phenylindole Dihydrochloride (DAPI, Life Technologies) and quantified using Image J software (National Institute of Health, USA). Briefly, the color image (RGB) was converted to grayscale (8-bit) and the threshold was adjusted. Watershed function was used to divide particles which had merged together. Nuclei were then counted, setting the size (pizel^2) between 400 and 3000 and circularity as default (0.00-1.00). The number of counted particles was then corrected for the area of the filter using Microsoft Excel Software.

Statistical analysis

All experiments were carried out in at least 3 independent replicates. Data are expressed as mean ± SEM. Analysis of the experimental data was performed by GraphPad Prism 5.0.

Results

Co-expressed exogenous B^0AT1 and TMEM27 localize to the luminal membrane of MDCK cells

To investigate the impact of amino acids on the expression of the major renal proximal tubule luminal sodium-neutral amino acid symporter B^0AT1-TMEM27, we established MDCK cells overexpressing both transporter subunits using a retroviral system. Such an *in vitro* system was successfully employed for previous studies on overexpressed luminal amino acid antiporter $b^{0,+}$AT-rBAT (SLC7A9-SLC3A1) and basolateral y^+LAT1-4F2hc (SLC7A7-SLC3A2) and LAT2-4F2hc (SLC7A8-SLC3A2) [8,9]. Immunofluorescence studies on polarized MDCK cells showed an apical co-localization of B^0AT1 and TMEM27 when co-expressed (Fig. 1A). Consistent with previous observations in other expression systems [16], TMEM27 co-expression increases B^0AT1 transport function also at the luminal surface of cultured MDCK cell epithelia (Fig. 1B).

Expression of exogenous B^0AT1 and TMEM27 is prevented by high amino acid content of cell culture medium and regulated upon mycoplasma infection

As previously observed in various attempts to (over)express B^0AT1 and TMEM27 in MDCK cells (data not shown), the expression of B^0AT1 and TMEM27 proteins strongly decreased following subsequent cell culture passages (Fig. 1C and 1D). This effect was specific for the amino acid transporter and its accessory protein as the control cell line overexpressing EGFP did show a less significant change in protein expression. We speculated that in the elevated extracellular amino acid concentrations in standard cell culture medium the concentrative transporter B^0AT1 increases intracellular amino acids concentrations. Increased intracellular levels exert a negative feedback on the activity and/or expression of the transporter, resulting in its downregulation. To test this hypothesis, freshly transduced B^0AT1-TMEM27 overexpressing MDCK cell lines were subcultured on plastic dishes in low

amino acids containing media (so called physiological medium) or in control media (classical DMEM) and transgene protein expression was assessed by Western blotting. Unexpectedly, physiological amino acid containing medium did not significantly increase B^0AT1-TMEM27 expression after 10 passages (Fig. S1). To test the effect of amino acid concentrations on polarized epithelia, B^0AT1-TMEM27 overexpressing MDCK cell lines were cultivated on filters in physiological or standard cell culture medium and transgene mRNA and protein expression was assessed by quantitative PCR and Western blotting, respectively. When comparing various B^0AT1-TMEM27 overexpressing MDCK cell lines under otherwise identical experimental conditions, we observed considerable variations in the response to physiological medium. PCR-based test for the presence of mycoplasma revealed an infection of these cell lines (data not shown) explaining their observed phenotypic change. Interestingly, B^0AT1-TMEM27 overexpressing mycoplasma-infected MDCK cells cultured in low amino acid media reproducibly demonstrated a rapid time-dependent increase of transgene mRNA (Fig. 2A and 2B) and protein expression (Fig. 2C and 2D), with a peak at three days. However, this effect of culture in low amino acid media was not specific for B^0AT1-TMEM27 expression. Indeed, mycoplasma-infected MDCK cell lines overexpressing EGFP or TMEM27 in the absence of B^0AT1 displayed a similar upregulation of transgene mRNA expression in low amino acid medium (Fig. S1). In contrast, the mRNA expression of endogenous housekeeping genes such as GAPDH and β-actin was unaffected (data not shown). Taken together, these results suggested that in low amino acid medium mycoplasma infection upregulates mRNA and protein expression of transgenes in MDCK cells.

Mycoplasma-mediated L-arginine depletion stimulates exogenous B^0AT1 and TMEM27 expression

Next, we evaluated the dose response effect of media amino acids on transgene expression in mycoplasma-infected B^0AT1-TMEM27 overexpressing MDCK cells. Western blotting analysis of protein samples prepared from cells cultured in different amino acid concentrations revealed a dose-dependent effect on B^0AT1 expression (Fig. 3A).

To identify whether a specific amino acid was responsible for the observed transgene upregulation, we divided the 20 proteinogenic amino acids into four groups and tested their effect on B^0AT1 expression. Western blotting experiments showed that elevated essential amino acids alone were able to reduce B^0AT1 protein expression as strongly as the control media (8-fold plasma amino acid concentrations which is similar to standard culture medium). In contrast, none of the other amino acid groups affected B^0AT1 protein abundance (Fig. 3B). Therefore, the five essential amino acids of the group were tested individually. Only arginine, of the tested amino acids, significantly regulated B^0AT1 protein abundance (Fig. 3C). These data showed that in mycoplasma-infected MDCK epithelia transgene upregulation by low amino acid media is dose dependent and suggested a pivotal role for arginine.

To investigate the specific role of arginine on transgene expression, time course and dose response experiments were performed (Fig. 4). Quantitative PCR analysis showed that low arginine concentration drastically increases B^0AT1 mRNA expression in a time-dependent manner (Fig. 4A), whereas transgene mRNA expression was inhibited by high arginine concentrations. Likewise but to a lesser extent, B^0AT1 protein abundance was also regulated by low arginine concentrations in a time-dependent manner (Fig. 4B and 4C). Thus, these data indicated that in mycoplasma-infected MDCK cells low arginine levels increase both transgene mRNA and protein expression.

Transgene expression is not regulated by mammalian arginine metabolism

The arginine effect on transgene expression appeared to have a relatively slow time course, suggesting the possibility that amino acid metabolism plays a critical role. We analyzed the amino acid concentration in the media of mycoplasma-infected MDCK cells cultured in low and high arginine media. Our data showed that arginine was indeed depleted from the medium within 24 h when initially administered at low concentration (Fig. 5A). Even when given at high concentration arginine metabolism led to its depletion within 48 h. This increased amino acid metabolism was arginine-specific as all other amino acids were not significantly decreased in the medium after 48 h (data not shown). Arginine consumption rate was correlated with ornithine production (Fig. 5B). Citrulline was also measured but its concentration was below the detection limits (<10 µM) at all the time points, except for the 48 h (7.1±3.9 µM in low arginine medium and 13.1±1.7 µM in high arginine medium).

Next, we tested whether inhibitors of mammalian arginine metabolizing enzymes, in particular of arginase, nitric oxide synthase and ornithine decarboxylase impact on B^0AT1 expression (see Fig. 6A for pathways). The fact that none of the tested drugs had an effect on B^0AT1 protein expression suggested that their products are not involved in the observed transgene regulation (Fig. 6 B-D). Consistent with this observation, incubation of the transduced MDCK cells with high doses of ornithine, urea, polyamines, citrulline or a NO donor also did not affect transgene expression (Fig. 6E and 6F). Furthermore, the effect on transgene expression seemed to be specific for L-arginine, as D-arginine did not exert any effect (Fig. 6E). Thus, these results suggested that the observed transgene upregulation was not mediated by products of L-arginine metabolism but by the lack of L-arginine.

GCN2 pathway is activated by low arginine levels in mycoplasma-infected MDCK cells

Recent studies have shown that transgene expression is regulated by low levels of essential amino acid such as Tyr and Met/Cys which trigger a downregulation of the histone deacetylase 4 (HDAC4) [27]. Indeed, inhibition of HDAC activity by trichostatin A (TSA) also upregulated the protein expression of B^0AT1 (Fig. 7A). However, the mRNA expression of HDAC 4 as well as that of others HDACs (2, 3, 5, 6, 8, 10 and 11) was not regulated by a decrease in arginine concentration (data not shown).

AA availability is known to be sensed by mTOR and the GCN2 pathway. To investigate whether mycoplasma-induced arginine depletion results in the regulation of one of these two pathways, the phosphorylation of their downstream effectors 4EBP-1 and eIF2α, respectively, was measured by Western blotting. While the phosphorylation of the mTOR downstream effector 4EBP-1 was unaffected by low arginine levels (Fig. 8A and 8B), the phosphorylation of the GCN2 effector eIF2α was rapidly increased after 4 h of incubation in low arginine medium (Fig. 8C and 8D). Interestingly, in the presence of high arginine medium eIF2α phosphorylation was also increased, but with a much slower time course. Taken together, these data showed that mycoplasma infection of MDCK cell cultures causes a rapid L-arginine depletion, which in turn activates the GCN2 pathway.

Mycoplasma-induced arginine depletion affects MDCK cell number and tight epithelium formation

The GCN2 pathway activation leads to a global decrease in protein synthesis, which in turn affects several vital processes such as proliferation, differentiation and apoptosis [5]. Indeed, mycoplasma-infected MDCK wild type cells showed a 20% reduction in cell number after 24 h culture in low arginine medium (Fig. 8A). In contrast, cells cultured in high arginine medium showed a progressive increase in cell number within 72 h. We also found that mycoplasma-infected MDCK cells cultivated on porous filters with low arginine medium

presented low trans-epithelial electrical resistance (TEER) during the time of culture (Fig. 8B). In contrast, MDCK cells cultured in high arginine medium showed an increase in TEER over time, compatible with the formation of epithelial tight junctions. To determine if the effect of arginine on trans-epithelial electrical resistance was due to the regulation of tight junction proteins expression, we performed immunofluorescence staining of ZO-1, a classical component of tight junctions [28]. Our data clearly showed that mycoplasma-infected MDCK cells initially had a low expression of ZO-1 at the tight junctions when treated with low arginine medium (Fig. 8C, t=24h). Nevertheless, both arginine treatments resulted in comparable ZO-1 protein abundance at the end of the cell culture (Fig. 8C, t=72h), suggesting that low arginine medium retards but does not fully prevent tight junction formation. These results showed that arginine deprivation dramatically affects MDCK epithelia, resulting in a decrease in cell number and TEER, which in turn corresponds to a delay in tight junction formation.

Discussion

The aim of this study was to characterize the impact of amino acids on the expression of the neutral amino acid transporter B^0AT1 and its accessory protein TMEM27 in MDCK cells. The rationale for this study was based on the observation that B^0AT1-TMEM27 overexpressing MDCK cells displayed progressively reduced transgene expression after only a few culture passages. Interestingly, infection of MDCK cells with mycoplasma led to a rapid arginine depletion, which in turn triggered the derepression of silenced transgenes, most likely through the activation of the GCN2 pathway. These findings expand the biochemical changes induced by mycoplasma to the metabolism of infected cells by adding the novel role for arginine in the control of transgene expression [21]. Transgene silencing represents a common defense mechanism of mammalian cells, which identify the foreign sequences as cellular invaders and target them for silencing. Recent studies have shown that limitation of essential amino acids such as Tyr or Met/Cys regulates transgene expression in mammalian cells [27]. Here, we show that another (conditionally) essential amino acid, namely arginine can affect transgene expression. However, these results are in variance with those of Palmisano et al. [27], who suggested that the transgene regulation is a process involving inactivation or downregulation of the histone deacetylase 4 (HDAC4). Although both studies agree that amino acid deprivation activates a general response able to derepress integrated transgenes, we could not confirm the regulation of the transgene by histone deacetylases. Instead, the decrease of arginine in the medium was correlated with an increase in eIF2α phosphorylation after 4 h, which suggests that the transgene regulation is mediated by the activation of the GCN2 pathway. The amino acid response induced by amino acid deprivation is known to regulate gene expression at many steps, from the chromatin structure to the transcription and translation rates [1,29]. However, the mechanism which controls chromatin structure remodeling in response to amino acid deprivation is not yet understood [1]. Interestingly, the expression of several genes

encoding transcription factors has been shown to be increased by amino acid limitation. Examples include members of the activating transcription factor (ATF) family, FOS/JUN family, CCAAT/enhancer binding protein (C/EBP) family, and other transcription factors outside of the bZIP superfamily (reviewed in Ref. [30]). To date, there are no known transcription factors that increase the CMV promoter transcription rate resulting in an increase of the mRNA expression of transgenes. Instead, luciferase constructs driven by CMV promoter have been shown to be unaffected by amino acid deprivation in skeletal muscle cells [31].

In the present study, inhibition of mammalian arginase, nitric oxide synthase and ornithine decarboxylase did not affect the transgene expression in MDCK cells. The rapid and massive breakdown of arginine to ornithine in MDCK cells is consistent with the time course previously observed in HeLa cells infected with mycoplasma [20]. Furthermore, arginine deiminase, the first mycoplasma enzyme involved in arginine breakdown, has a much higher arginine affinity (Km ~ 30 µM) than mammalian arginases (Km ~ 45 mM) [32]. Thus, although arginase activity of the cell line could account for some ornithine production, it is unlikely that the observed arginine degradation is an intrinsic feature of MDCK cells, but more likely it is an effect of mycoplasma infection. Given that the reaction which converts arginine to ornithine has a stoichiometry of 1, it was surprising to observe that ornithine concentration in the low arginine medium exceeded by far the expected concentration. It is possible that part of measured ornithine derives from the metabolism of glutamine and glutamate, which are present at approximately 6 mM in the cell culture medium.

In addition to the effect on transgene expression, we found that mycoplasma-induced arginine depletion has an overall impact on MDCK epithelium formation. The reduction in MDCK cell number is consistent with previous studies which showed that deprivation of

essential amino acids from the cell culture medium (including arginine) triggers cell death [33,34]. Interestingly, cells treated with high arginine concentrations increased in cell number. However, the phosphorylation of 4E-BP1 protein was not affected by arginine levels in the media, suggesting that the mTOR pathway does not respond to arginine under the tested conditions in MDCK cells. These findings differ from previous studies which showed that arginine regulates the mTOR effectors in intestinal cells [35,36]. This is either due to a cell line difference or to the arginine concentration that we used, which might not have been sufficient to trigger the signaling pathway. Furthermore, we measured surprisingly low values of TEER in cells cultivated with low arginine medium. This might be a direct consequence of the lower cell number or of maturational changes in tight junction proteins.

Taken together, we propose that the lack of arginine induced by mycoplasma infection is sensed by MDCK cells and triggers GCN2 pathway activation, which in turn results in exogenous gene reactivation. The proposed GCN2-dependent mechanism responsible for the derepression of silenced transgenes opens the door to new approaches aiming at improving viral-mediated gene therapy strategies for the treatment of genetic disorders and acquired diseases.

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Figures

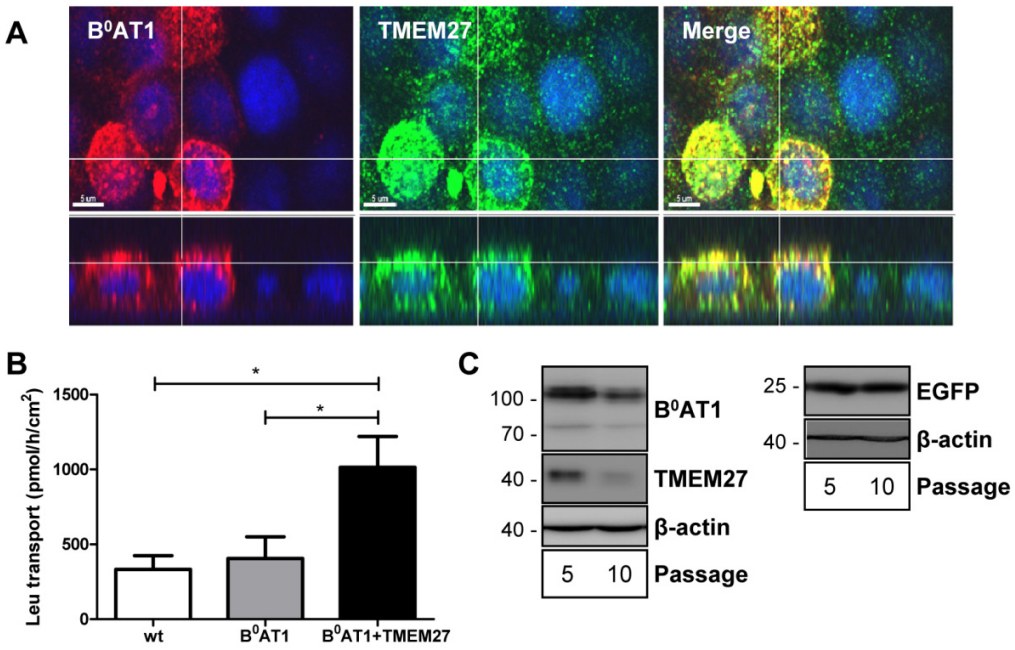

Figure 1. Overexpression of B^0AT1 and TMEM27 in MDCK cells. A: immunofluorescence analysis of B^0AT1-TMEM27 overexpressing MDCK cells. Apical co-localization (yellow) of B^0AT1 (red) and TMEM27 (green) is visualized by confocal microscopy. Upper panels represent images taken parallel to the filter (x-y plane); lower panels show corresponding z-x reconstitutions. Bars = 5 µm. B: Apical uptake of L-Leu (100 µM, 10 min) in wild type (wt) or B^0AT1 or B^0AT1-TMEM27 overexpressing MDCK cells cultivated on filters. Data are represented as mean ± SEM (n=3). Groups were compared by one-way ANOVA followed by Tukey post-test; * $p \leq 0.05$. C: MDCK cells overexpressing B^0AT1-TMEM27 or EGFP. After viral transduction, cells were subcultured on plastic dishes for 5 or 10 times (passage 5 and 10, respectively) in standard cell culture medium. Western blotting experiments with antibodies directed against B^0AT1, TMEM27 and EGFP were performed. Representative Western blotting images are shown.

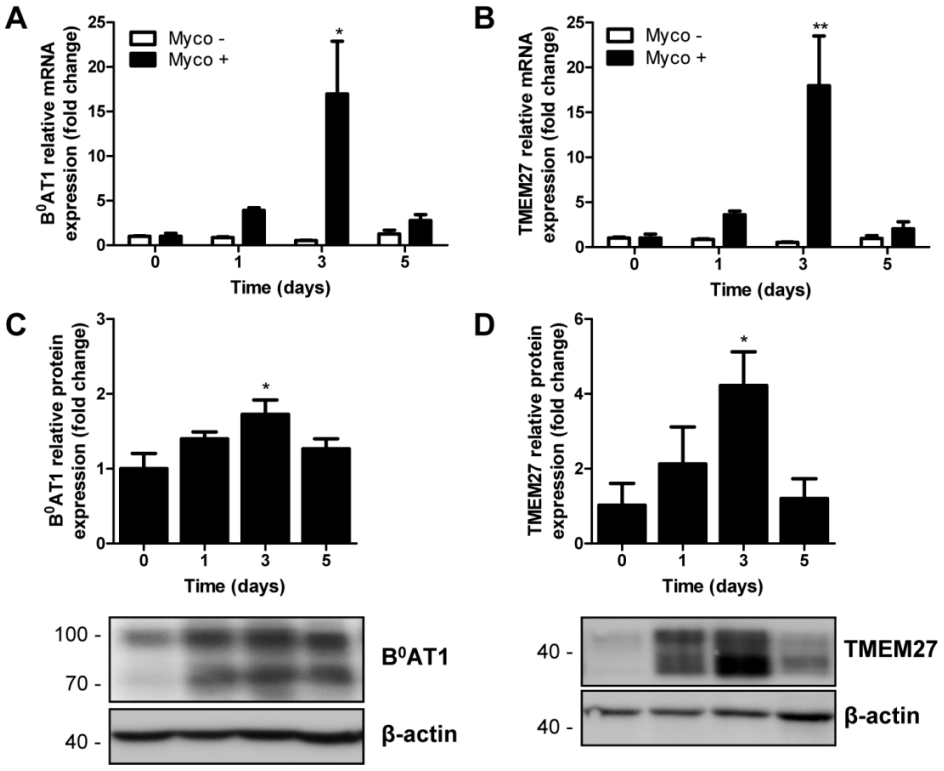

Figure 2. Effect of physiological amino acid levels on transgenes expression. Cells were cultivated on filters in standard cell culture medium and treated for the indicated times with physiological medium. A-B: mRNA expression of B^0AT1 (A) or TMEM27 (B) was measured by quantitative PCR-analysis in B^0AT1-TMEM27 overexpressing MDCK cells which were found Mycoplasma-free (open bar) or mycoplasma-infected (black bar). mRNA levels were standardized to β-actin and normalized to time 0. C-D: Western blotting experiments with antibodies directed against B^0AT1 (C) or TMEM27 (D) were performed in mycoplasma-infected B^0AT1-TMEM27 overexpressing MDCK cells and the intensity of the immunoreactive bands was quantified, standardized to β-actin and normalized to time 0. Representative Western blotting images are shown. Data are represented as mean ± SEM (n=3). Groups were compared by one-way ANOVA followed by Dunnett post-test; ** $p \leq 0.01$, * $p \leq 0.05$.

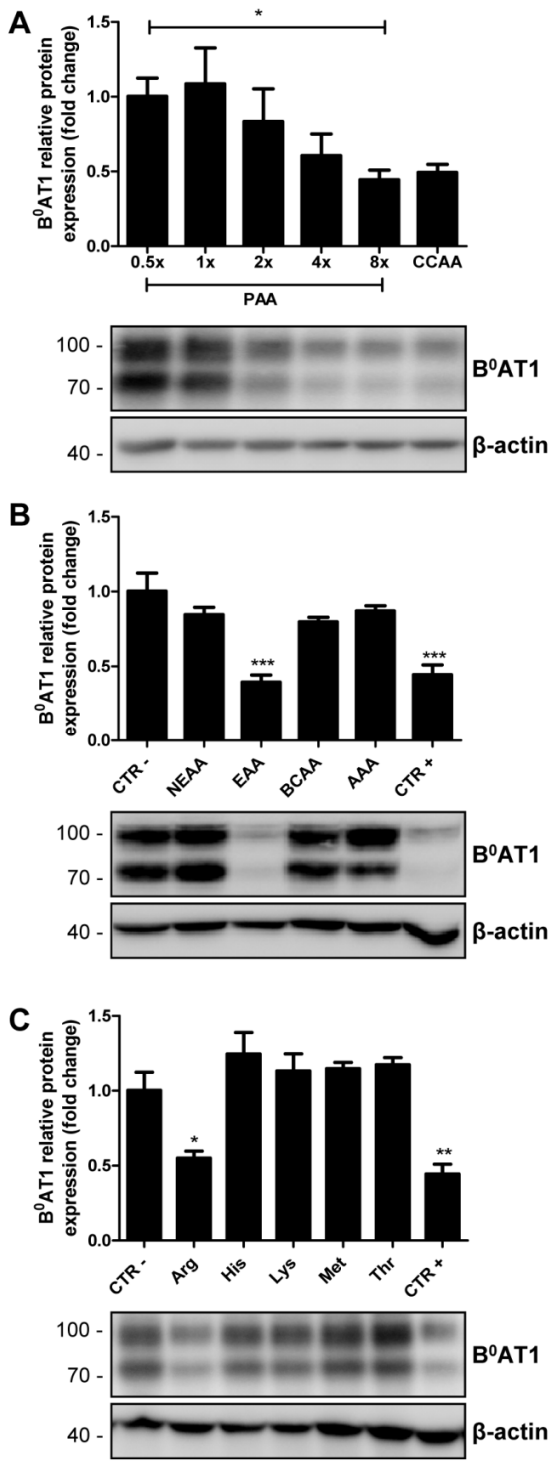

Figure 3. Transgene regulation by amino acids. A: Mycoplasma-infected B^0AT1-TMEM27 overexpressing MDCK cells were incubated in standard cell culture medium (CCAA) or in amino acid-free medium complemented with different amounts (0.5x to 8x) of an amino acid mixture corresponding to the concentrations found in post-absorptive mouse plasma (PAA doses). B^0AT1 expression was analyzed by Western blotting and the intensity of the immunoreactive bands was quantified, standardized to β-actin and normalized to the level measured in 0.5 fold complemented medium. B-C: Mycoplasma-infected B^0AT1-TMEM27 overexpressing MDCK cells were incubated in a modified medium containing the indicated groups (B) or single (C) amino acids (NEAA: non essential amino acids – Ala, Asn, Asp, Cys, Glu, Gln, Gly, Pro and Ser; EAA: essential amino acids – Arg, His, Lys, Met and Thr; BCAA: branched chain amino acids – Ile, Leu and Val; AAA: aromatic amino acids – Phe, Trp and Tyr) at 8x their normal plasma concentrations whereas the remaining amino acids were given at 0.5x their normal plasma level. CTR - and + represent cells which were treated with all the amino acids at 0.5x and 8.0x their plasma level, respectively. B^0AT1 expression was analyzed by Western blotting and intensity of the immunoreactive bands was quantified, standardized to β-actin and normalized to CTR -. Representative Western blotting images are shown. Data are represented as mean ± SEM (n=3). Groups were compared by one-way ANOVA followed by post-test for linear trend (A) or Dunnett post-test (B-C); *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$.

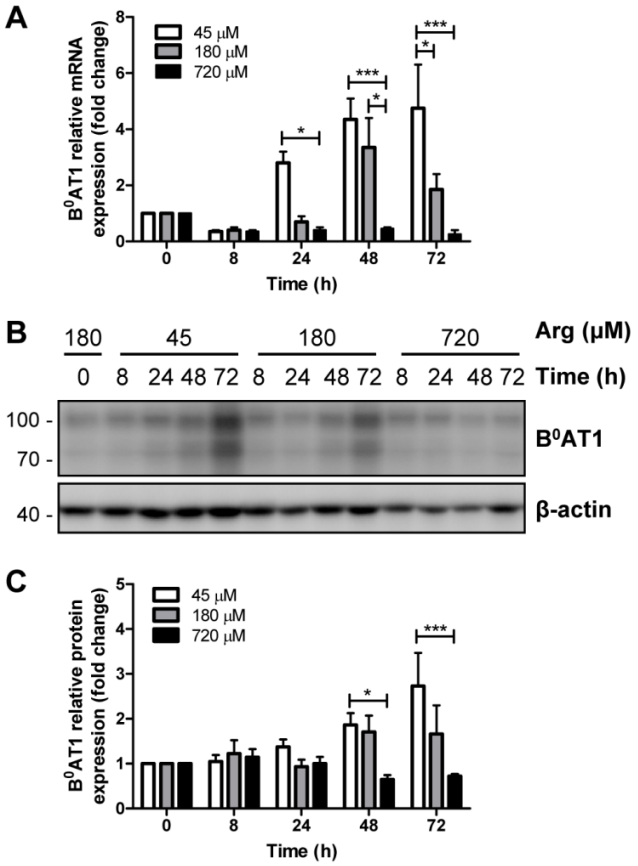

Figure 4. Time course and concentration dependence of effect of arginine on transgene expression. Mycoplasma-infected B^0AT1-TMEM27 overexpressing MDCK cells were cultivated in arginine-free DMEM supplemented with 45, 180 or 720 μM arginine for the indicated times. A: Real-time PCR quantification of B^0AT1 mRNA expression. mRNA levels were standardized to β-actin and normalized to time 0. B: Representative Western blotting of B^0AT1 expression. C: the intensity of B^0AT1 immunoreactive bands was quantified, standardized to β-actin and normalized to 0 h. Data are represented as mean ± SEM (n=3). Groups were compared by two-way ANOVA followed by Bonferroni post-test; *** $p \leq 0.001$, * $p \leq 0.05$.

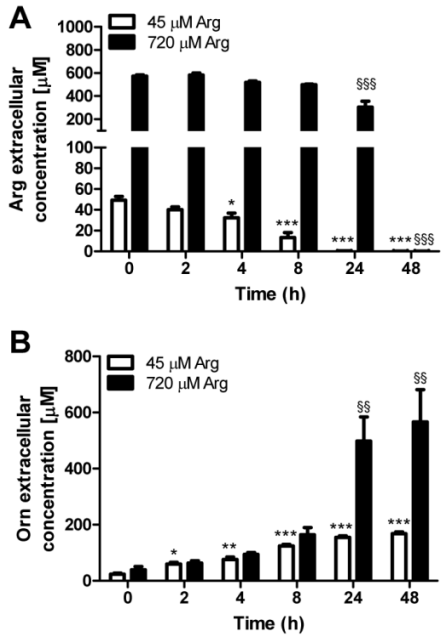

Figure 5. Time course of arginine metabolism in low and high arginine medium. Concentrations of arginine (A) and ornithine (B) were measured in the medium of mycoplasma-infected B^0AT1-TMEM27 overexpressing MDCK cells treated with 45 µM (open bar) or 720 µM (black bar) arginine at the indicated times. Groups were compared by one-way ANOVA followed by Dunnett post-test; *** p≤ 0.001, ** p≤ 0.01, * p≤ 0.05 versus 45 µM arginine; §§§ p≤ 0.001, §§ p≤ 0.01, versus 720 µM arginine.

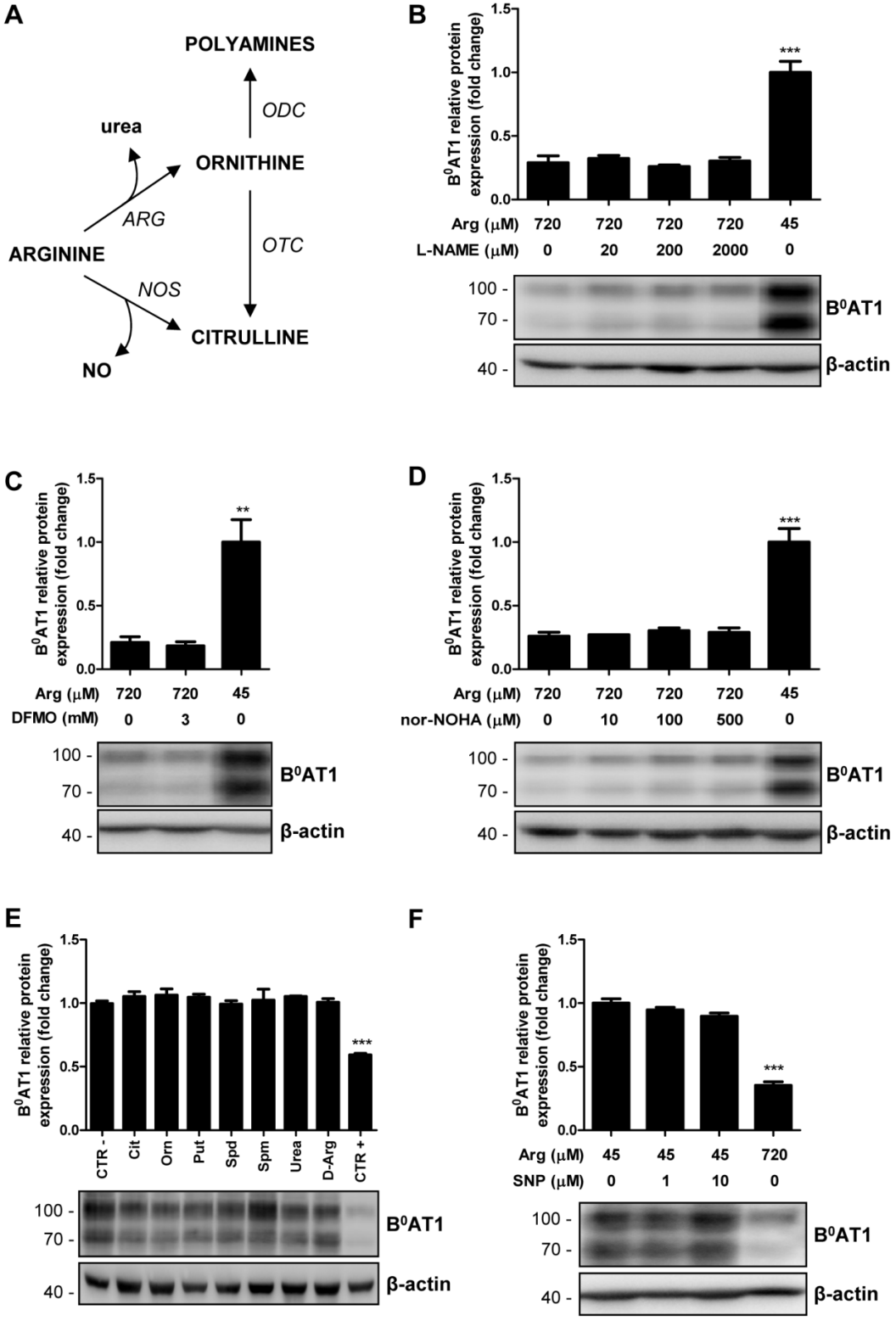

Figure 6. Effect of L-arginine metabolism on transgene expression. A: Schematic overview of some mammalian arginine metabolic pathways. ARG: arginase; NO: nitric

oxide; NOS: nitric oxide synthase; ODC: ornithine decarboxylase; OTC: ornithine transcarbamylase. B-D: Mycoplasma-infected B^0AT1-TMEM27 overexpressing MDCK cells were cultivated in arginine-free DMEM supplemented with 45 or 720 µM arginine in the presence or absence of the indicated concentrations of inhibitors of the following enzymes: (B) nitric oxide synthase (N^G-nitro-L-arginine methyl ester, L-NAME); (C) ornithine decarboxylase (α-difluoromethylornithine, DFMO) and (D) arginase (Nw-Hydroxy-nor-L-arginine, nor-NOHA). E-F: Mycoplasma-infected B^0AT1-TMEM27 overexpressing MDCK cells were cultivated in arginine-free DMEM supplemented with 45 µM arginine in the absence (negative control, CTR -) or presence of (E) the indicated metabolites or (F) a nitric oxide donor (sodium nitroprusside, SNP). Positive control (CTR +) represents cells treated with 720 µM arginine. B^0AT1 expression was analyzed by Western blotting and intensity of the immunoreactive bands was quantified, standardized to β-actin and normalized to 45 µM arginine. Representative Western blotting images are shown. Data are represented as mean ± SEM (n=3). Groups were compared by one-way ANOVA followed by Dunnett post-test; *** $p \leq 0.001$ ** $p \leq 0.01$.

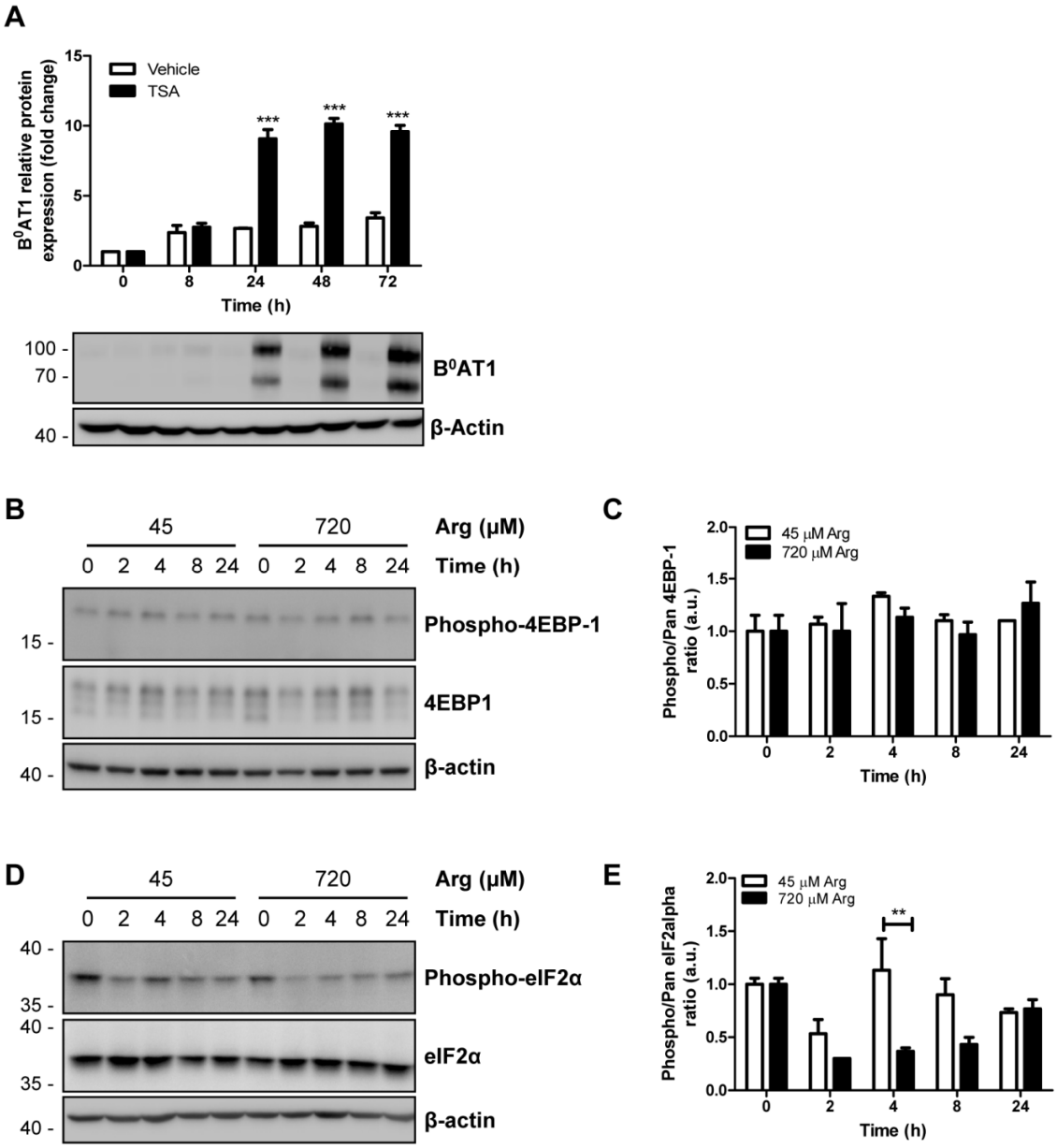

Figure 7. Effect of arginine on cell signaling. A: Mycoplasma-infected B^0AT1-TMEM27 overexpressing MDCK cells were grown in standard cell culture medium supplemented with an inhibitor of histone deacetylation (TSA, 1 μM) for the indicated times. B^0AT1 expression was analyzed by Western blotting and intensity of the immunoreactive bands was quantified, standardized to β-actin and normalized to time 0. Representative Western blotting images are shown. Data are represented as mean ± SEM (n=3). Groups were compared by two-way ANOVA followed by Bonferroni post-test; *** p≤ 0.001. B-E: Mycoplasma-infected B^0AT1-TMEM27 overexpressing MDCK cells were incubated with 45 or 720 μM arginine medium for the indicated times and the whole cell protein extracts were

subjected to immunoblotting for total- and phospho-4EBP-1 (B-C) and eIF2α (D-E). Representative Western blotting images are shown in B and D. The degree of 4EBP-1 (C) and eIF2α (E) phosphorylation was assessed by quantifying the immunoreactive bands of the phosphorylated form and normalizing to the total protein in each lane. Data are represented as mean ± SEM (n=3). Groups were compared by one-way ANOVA followed by Dunnett post-test; ** $p \leq 0.01$.

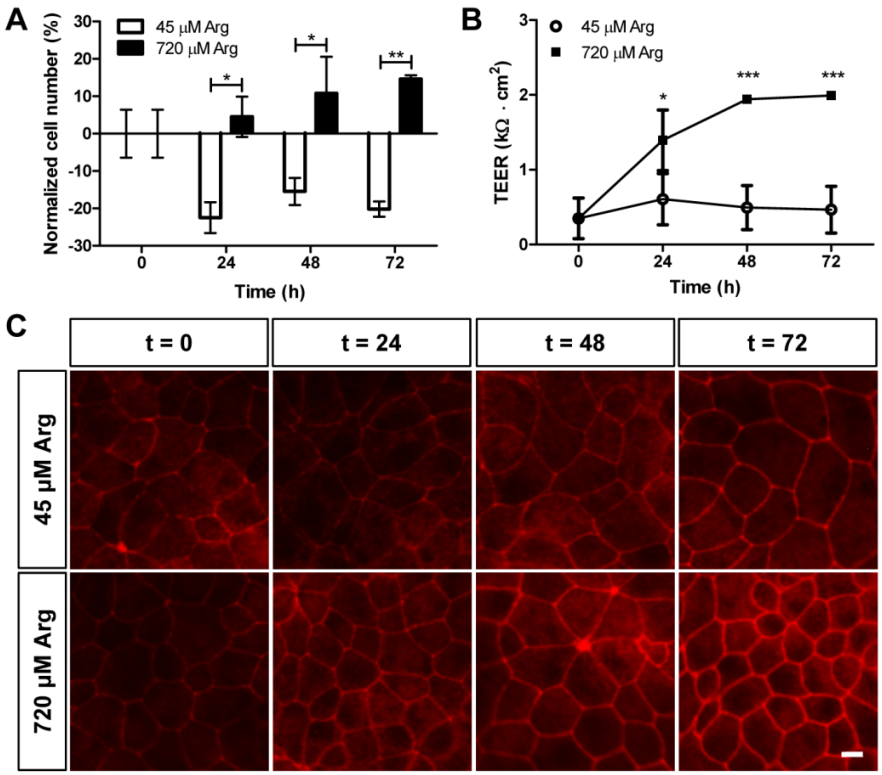

Figure 8. Effect of arginine on cell number and tight epithelium. Mycoplasma-infected MDCK wild type (wt) cells were cultivated in arginine-free DMEM supplemented with 45 or 720 µM arginine for the indicated times. A: Cell number was estimated based on DAPI-staining followed by Image J analysis as described in Materials and Methods. B: Trans-epithelial electrical resistance (TEER) was measured every 24 h using EVOHM device. Data in panels A and B are represented as mean ± SEM (n=3). Groups were compared by two-way ANOVA followed by Bonferroni post-test; *** p≤ 0.001, ** p≤ 0.01, * = p≤ 0.05. C: Immunofluorescence analysis of representative wt MDCK cells with antibodies raised against the tight junction protein ZO-1. Scale bar is 10 µm and applies to all panels.

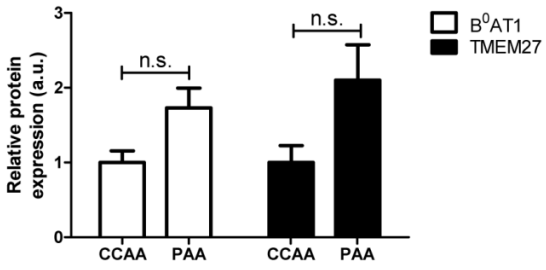

Figure S1. Effect of physiological amino acid levels on B^0AT1-TMEM27 overexpressing MDCK cell cultures. After viral transduction, B^0AT1-TMEM27 overexpressing MDCK cells were subcultured on plastic dishes for 10 passages either in standard cell culture medium (CCAA) or in physiological medium (PAA). Western blotting experiments with antibodies directed against B^0AT1 and TMEM27 were performed. The intensity of the immunoreactive bands was quantified, standardized to β-actin and normalized to CCAA. Data are represented as mean ± SEM (n=3). No significance was observed when groups were compared by unpaired two-tailed t-test.

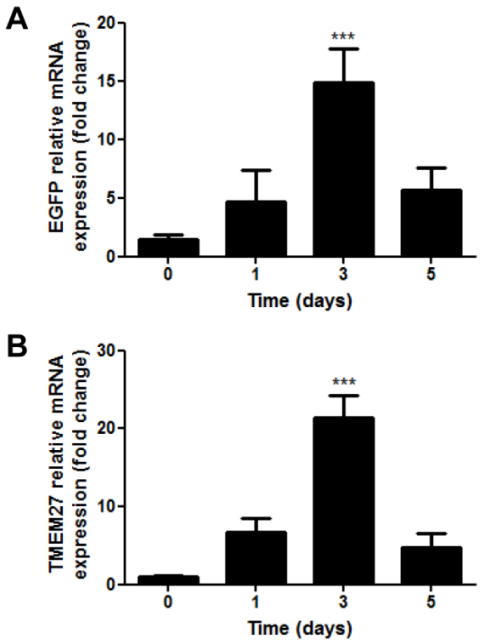

Figure S2. Effect of physiological amino acid levels on expression of other transgenes. A-B: Mycoplasma-infected MDCK cells overexpressing EGFP (A) or TMEM27 (B) were cultivated on filters in standard cell culture medium and treated for the indicated times with physiological medium. Quantitative RT-PCR analysis was performed and mRNA levels were standardized to 18S and normalized to time 0. Data are represented as mean ± SEM (n=3). Groups were compared by one-way ANOVA followed by Dunnett post-test; *** $p \leq 0.001$.

Tables

Amino Acid	Cell culture medium	Physiological medium
Glycine	500	250
L-Alanine	100	320
L-Arginine Hydrochloride	398	90
L-Asparagine	100	50
L-Aspartic Acid	100	5
L-Cystine Dihydrochloride	201	30
L-Glutamic Acid	100	30
L-Glutamine	5973	660
L-Histidine Hydrochloride-H_2O	200	90
L-Isoleucine	802	60
L-Leucine	802	120
L-Lysine Hydrochloride	798	200
L-Methionine	201	25
L-Phenylalanine	400	50
L-Proline	100	160
L-Serine	500	110
L-Threonine	798	130
L-Tryptophan	78	90
L-Tyrosine	398	60
L-Valine	803	220

Table 1. Amino acid concentrations (μM) in cell culture and physiological media.

Target mRNA	Accession number	Sequence (5'→3')
β-Actin	NM_001195845.1	Sense: CAAGGTTGGGGACTTAGCTG Antisense: GAGTAGAGTGAGCATGAGATCCAG Probe: #97 (Cat no. 04692144001, Roche)
B^0AT1	NM_001003841.2	Sense: GTGTGGACAGGTTCAATAAGGACAT Antisense: CCACGTGACTTGCCAGAAGAT Probe: TCATGATCGGCCACAAGCCCAA
TMEM27	NM_020665.5	Sense: CCTCTTCAAAGCGATGGTAGCT Antisense: CCCTCTGGGTTACATTGCAAA Probe: CCCAACAGAGAAGCAACAGAAATTTCCCA
eGFP	YP_002302326.1	Sense: GTCCGCCCTGAGCAAAGA Antisense: TCCAGCAGGACCATGTGATC Probe: CCCAACGAGAAGCG

Table 2. List of primers and probes used for quantitative real-time PCR.

6. DISCUSSION AND OUTLOOK

6.1 Summary of results

The initial aim of this thesis was to understand the cellular and molecular mechanism underlying B^0AT1 and TMEM27 interaction. To achieve this goal we generated MDCK cells overexpressing B^0AT1 and/or TMEM27. Our data showed that TMEM27 increases B^0AT1 function by upregulating its surface expression.

In the course of this study, we noticed that B^0AT1-TMEM27 protein expression strongly decreased following subsequent cell culture passages. This effect was specific for the amino acid transporter and its accessory protein as the control cell line overexpressing EGFP did show a less significant change in protein expression. We speculated that the elevated amino acid concentrations in standard cell culture medium exert a negative feedback on the activity and/or expression of B^0AT1-TMEM27, resulting in its downregulation. Unexpectedly, B^0AT1-TMEM27 protein expression was unaffected by physiological amino acid concentrations in the media. Interestingly, mycoplasma infection resulted in a significant increase in transgene expression and correlated with the rapid metabolism of L-arginine. However, L-arginine metabolites were shown to play no role in transgene expression. Instead, we observed a rapid increase in eIF2α phosphorylation which suggested that activation of the GCN2 pathway may trigger transgene derepression. In addition to the transgene regulation, we found that mycoplasma-induced arginine depletion dramatically affected MDCK epithelia, resulting in a decrease in cell number and trans-epithelial electrical resistance corresponding in a delay in tight junction formation.

6.2 Outlook

6.2.1 MDCK cell model: inducible or constitutive system?

In the first part of this thesis we employed an MDCK cell model overexpressing B^0AT1 and TMEM27 under the control of an inducible promoter. This system was used for a preliminary characterization of the role of TMEM27 in B^0AT1 function and expression. However, the model showed several disadvantages (uneven co-expression of the transgenes, loss of transgene expression along cell culture passages, endogenous expression of TMEM27 and ACE2) which limited the study and the interpretation of the results (see also discussion 4.3.1). To obtain a more uniform B^0AT1-TMEM27 co-expression, we designed a retroviral IRES-containing bicistronic vector which allowed the simultaneous expression of the two proteins from the same RNA transcript. MDCK cells transduced with this construct were successfully employed later in this study (see manuscript Torrente et al.) and showed higher B^0AT1-TMEM27 co-expression when compared to the previously used inducible system. However, B^0AT1-TMEM27 protein expression was still rapidly decreased along cell culture passages and could not be rescued by lowering amino acid concentrations in the cell culture medium. In summary, B^0AT1-TMEM27 constitutively overexpressing MDCK cells may represent a good model for studying the mechanism of B^0AT1-TMEM27 interaction. However, the experiments should be performed only with low passage cells, as the expression of both transgenes is lost during cell culture passages. Furthermore, the endogenous expression of TMEM27 and ACE2 should be silenced in order to better understand the role of exogenous TMEM27 in B^0AT1 trafficking to the apical membrane.

6.2.2 Loss of B^0AT1-TMEM27 (over)expression in MDCK cells

The reason for the loss of B^0AT1-TMEM27 protein expression in MDCK cells following subsequent cell culture passages remains unclear. The fact that the expression of other gene products such as various amino acid antiporters (previously studied by Bauch et al., 2002 and 2003) and EGFP (used as control in this study) was much more stable suggested a selective downregulation of the concentrative amino acid transporter B^0AT1-TMEM27. Interestingly, inhibition of the

HDACs by TSA resulted in an increase in transgene expression (Fig. 7A from the manuscript Torrente et al.). These data suggested that the transgene silencing is due to epigenetic modifications rather than transgene loss. To test this hypothesis, the effect of HDAC inhibitors in B^0AT1-TMEM27 overexpressing MDCK cells cultivated at different passages should be assessed. Future studies should also investigate the effect of intracellular amino acid concentrations on transgene expression. Our data showed that mycoplasma-induced arginine deprivation induces transgene derepression. Similarly, previous studies demonstrated that deprivation of other essential amino acids, namely Tyr and Cys/Met, leads to transgene upregulation (Palmisano et al., 2012). Thus, amino acid concentrations play a role in transgene expression regulation. If indeed high intracellular amino acid levels induce transgene repression, we could explain the rapid downregulation of the concentrative amino acid transporter B^0AT1-TMEM27. To address this question, experiments with MDCK cells overexpressing B^0AT1 mutants which reach the plasma membrane but remain inactive (Camargo et al., 2009) should be performed.

6.2.3 What is the role of TMEM27 association to B^0AT1?

The results presented in this thesis confirmed the importance of TMEM27 for B^0AT1 surface expression and function. However, the cellular and molecular mechanism of their interaction remains still unclear. The physical association of TMEM27 to B^0AT1 has not been demonstrated *in vitro* (see discussion 4.3.2). Co-immunoprecipitation experiments should be repeated with B^0AT1-TMEM27 constitutively overexpressing MDCK cells cultivated at low passage in order to guarantee high levels of expression of both transgene proteins. Furthermore, different immunoprecipitation conditions (ionic strength of the buffer, detergents, etc.) should be tested. It is also to be investigated whether this interaction is direct or rather mediated by other proteins, possibly by SNARE's. Similarly to the co-immunoprecipitation experiments, pulse-chase analysis should also be repeated with a more appropriate cell model (see 6.2.1) in order to understand the effect of TMEM27 on B^0AT1 protein stability (see discussion 4.3.2). Finally, the pharmacological block of lysosomal or proteasomal degradation could identify which of these pathways might be relevant regarding B^0AT1 protein degradation.

6.2.4 Renal selective interaction of B^0AT1 with TMEM27

Even though both TMEM27 and ACE2 are expressed in renal proximal tubules (Singer et al., 2009), B^0AT1 interacts only with TMEM27 in the kidney. The reason for this preferential association remains unclear. One hypothesis is that ACE2 has another partner protein in proximal tubule cells. This association could prevent ACE2 from interacting with B^0AT1, as well as with other SLC6 amino acid transporters. Another possibility is that ACE2 requires a specific protein for its interaction with the SLC6 amino acid transporters. This protein might be expressed in the intestine and absent in the kidney. One strategy to determine putative ACE2 binding proteins could be to isolate complexes from renal and intestinal lysates using anti-ACE2 antibodies, followed by mass spectrometry analysis. The identified binding partners could then be subsequently verified by co-immunoprecipitation experiments. Recent studies showed that indeed in the small intestine ACE2 forms a complex with other proteins including B^0AT1 (Fairweather et al., 2012)

6.2.5 Is B^0AT1 regulated by amino acids *in vivo*?

The initial hypothesis that B^0AT1 expression could be regulated by amino acids *in vitro* was not confirmed in MDCK cells. The subsequently observed rapid upregulation of B^0AT1 mRNA and protein expression (within few days) upon culture in low amino acid containing medium was shown to be caused by mycoplasma-induced arginine depletion and affected also other tested transgenes. The regulation of amino acid transporter by amino acids remains an interesting and physiologically very relevant question. Indeed, protein diets have been shown to affect the expression of intestinal peptide transporters (i.e. PepT1) and amino acid transporters (i.e. EAAC1/SLC1A1) *in vivo* (Erickson et al., 1995; Shiraga et al., 1999). Furthermore, expression of the glucose transporter SGLT1 and PepT1 has been shown to be submitted to circadian regulation and food entrainment (Pan et al., 2004; Qandeel et al. 2009). Future studies should characterize the short- and long-term effect of protein diets on renal and intestinal B^0AT1 expression and function in *in vivo* models. Circadian regulation should also be taken into account when investigating the regulation of this amino acid transporter.

6.2.6 Transgene expression regulation by GCN2 pathway

The present study showed that mycoplasma-induced arginine deprivation triggers the increase in eIF2α phosphorylation in MDCK cells. Therefore, we suggested that transgene regulation is mediated by the activation of the GCN2 pathway. The GCN2 kinase is activated upon any amino acid limitation (Wek et al., 1995). It would be interesting to determine whether single amino acid deprivation increases transgene expression in mycoplasma-free MDCK cells. Furthermore, the mechanism regulating transgene transcription in mycoplasma-infected MDCK cells should be further investigated.

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8. CURRICULUM VITAE

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EDUCATION

03/2009 - present (Expected 03/2014)	**University of Zurich (Switzerland)**, *PhD in Integrative Molecular Medicine* Area of research: Cellular and molecular physiology of the kidney
10/2006 – 12/2008	**University of Padua (Italy)**, *Master of Science in Industrial Biotechnology* Focus: Advanced chemistry, Immunology
10/2003 – 07/2006	**University of Padua (Italy)**, *Bachelor in Biotechnology* Focus: Biology, Chemistry, Biochemistry

EXPERIENCE

03/2009 - present **University of Zurich (Switzerland)**
RESEARCH ASSISTANT. Institute of Physiology
- Worked on multiple research projects
- Developed experimental and analytical techniques to generate and process scientific data
- Presented at the international conferences for renal research and experimental biology
- Supervised practical laboratory classes for medical students
- Wrote scientific abstracts, reports and manuscripts

01/2008 – 11/2008 **University of Regensburg (Germany)**
UNDERGRADUATE RESEARCHER. Department of Pharmaceutical Technology
- Developed cell-based assays
- Conducted weekly update sessions with stakeholders

09/2007 – 01/2008 **University of Padua (Italy)**
PART-TIME ADMINISTRATIVE ASSISTANT. Faculty of Economics
- Organized computer lab access database
- Provided IT support during lectures and graduation ceremonies
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RESEARCH SKILLS

- *In vitro* cell culture techniques (immortalized and primary cell lines, establishing, maintenance, gene expression, viral transduction, assays development)
- Molecular biology (Cloning, mutagenesis, DNA and RNA extraction and purification, quantitative PCR, design of the gene constructs)
- Biochemistry (Protein expression and purification, SDS-PAGE, Western blotting, Immunoprecipitation, Protein co-precipitation, *In vitro* protein biotinylation)
- Radio-biology (radioimmunoassay, handling research radio-isotopes)
- Statistical analysis
- Scientific writing
- Data presentation

OTHER SKILLS

Computer: Windows, MS Office, Adobe PS, Acrobat, Graph-Pad Prism, Image J
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PUBLICATIONS AND CONFERENCES

- M.Torrente, A. Guetg L. Arps, L. Rückstuhl, S.M.R. Camargo and F. Verrey. "*Amino acids regulate transgene expression in MDCK cells*". Manuscript in preparation;
- M. Torrente, L. Arps, A. Guetg, S.M.R. Camargo and F. Verrey. *"Broad range neutral amino acid transporter (B^0AT1) requires association with TMEM27 for surface expression in renal cells"*. Abstract, oral and poster presentation at "Experimental Biology 2012" meeting in San Diego, California;
- M. Torrente, L. Arps, S.M.R. Camargo and F. Verrey. *"The broad range neutral amino acid transporter (B^0AT1) associates with collectrin in kidney cells"*. Abstract and poster presentation at "ZIHP 2010" meeting in Zurich, Switzerland;
- M. Torrente, L. Arps, S.M.R. Camargo and F. Verrey *"Broad range neutral amino acid transporter (B^0A* (Misfeldt et al., 1976)*T1) requires association with collectrin for surface expression in kidney cells"*. Abstract, oral and poster presentation at "Renal week 2010" in Denver, Colorado;
- M. Torrente, L. Arps, A.K. Behera, L. Rückstuhl, T. Dauwalder, S.M.R. Camargo, T. Ramadan and F. Verrey. *"Expression of the neutral amino acid transporter B^0AT1 (Slc6a19) and its specific renal accessory protein collectrin in MDCK cell line".* Abstract and poster presentation at "ZIHP 2009" meeting in Zurich, Switzerland.

FELLOWSHIPS AND AWARDS

Hartmann Müller-Stiftung travel grant (2012); Swiss Physiology Society travel grant (2011); Erasmus exchange program grant (2007); summa cum laude for Master of Science studies

INTERESTS AND ACTIVITIES

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